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CELLULAR IMMUNE RESPONSE (DTH) AGAINST A TUMOR-ASSOCIATED GLYCOPROTEINAND ITS SYNTHETIC CARBOHYDRATE EPITOPES.

BY

CARINA M. HENNINGSSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (IMMUNOLOGY)

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance a thesis entitled CELLULAR IMMUNE RESPONSE (DTH) AGAINSTA TUMOR-ASSOCIATED GLYCOPROTEIN AND ITS SYNTHETIC CARBOHYDRATE EPITOPES submitted by CARINA M. HENNINGSSON in partial fulfilment of the requirements for the degree of a Ph.D. in Medical Science (Immunology).

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Abstract:

We have attempted to determine the ability of T cells to recognize and respond to carbohydrate antigens by using a tumor associated glycoprotein, epiglycanin (epi) and synthetic carbohydrate haptens identical to known determinants on epi. The TF(a) (B-Gal-(1-3-)-a-GalNAc) and Tn (a-GalNAc-O-Serine), immunodominant determinants on epi were synthesized, conjugated to a carrier, HSA or KLH, and used to trigger a DTH response generated to epi. A Thy1+ Lyt1+ Lyt2- cell population primed with epi was elicited in a local DTH reaction by epi and the synthetic haptens linked to HSA or KLH but not by the HSA carrier alone nor by an inappropiate carbohydrate hapten such as Le^a linked to KLH. T cells of similar specificity were induced by synthetic antigens. The triggering of the local DTH reaction is dependent on macrophage antigen presentation in an H-2 restricted fashion.

Effector cells generated to either epi, synthetic TF, or synthetic Tn were able to specifically delay the growth of the TA3Ha tumor in a local Winn assay. The inhibition of the tumor growth was dependent on a Thy1+ Lyt1+ Lyt2- cell population, similar to the DTH reaction.

Direct in vivo immunization using epi or synthetic antigens led to a state of protection from tumor growth following a live tumor challenge. The in vivo induced protection was specific, but did not correlate directly with cell mediated immunity (DTH) or with humoral immunity.

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LIST OF ABREVIATIONS

HSA human serum albumin

BSA bovine serum albumin

KLH keyhole limp hemocyanin

DTH delayed type hypesensitivity

MHC major histocompatibility complex

RBC red blood cells

NERBC neuraminadase treated RBC

TAG tumor associated glycoconjugates

sTAG synthetic TAG

GalNAc NAcetyl-Galactosamine

Le^a $Lewis^a$ blood group antigen

MLR mixed leucocyte reaction

CTL cytotoxic lymphocyte

FCS fetal calf serum

NMS normal mouse sera

FPLC Fast pressure liquid chromotagraphy

PNA Peanut agglutinin

CFA complete Freund's adjuvant

ip intraperitoneal

iv intraveinously

ug microgram (10^6 gram)

ul microliter (10^6 liter)

Chapter 1:

Literature Review

TUMOR ASSOCIATED ANTIGENS.

Recent developments in tumor immunology have renewed interest in the potential of immunotherapy for the treatment of cancer. The rationale for tumor immunology and immunotherapy is based on three assumptions: (1) existence of tumor associated or specific antigens towards which an immune response can be directed, (2) that tumor growth reflects an inadequate or inappropriate immune response and (3) that the restoration of an appropriate immune response will correct the problem. Discussed below is the evidence from animal experimentation and clinical observation to support these assumptions.

Ehrlich first suggested that there could be antigenic differences between normal and neoplastic cells, such that it might be possible to immunize against cancer. Tumor immunology then fell into disrepute as it became clear that in the early studies the rejection of the tumor graft was due to a reaction against histocompatibility antigens. The development of inbred strains of animals in the 1950's allowed the demonstration that tumor grafts can be rejected by reactions against tumor-specific antigens. The existence of specific tumor antigens on methylcholanthrene -induced tumor cells was suggested when the tumor cells were rejected by a syngeneic host (1). The demonstration that immunization with tumor cells protected against a tumor graft but immunized against neither syngeneic

skin grafts, nor other unrelated tumors, provided further evidence in favor of tumor specific antigens (2). The demonstration that a tumor is immunogenic in the animal in which it originated provided unambiguous evidence for tumor associated antigens. Methylcholanthrene was used to induce a tumor in the leg of a mouse. The leg together with the tumor was then amputated and the tumor transplanted to a syngeneic host. The tumor was grown, and later irradiated tumor cells were used to immunize the animal in which it originated. A subsequent live tumor challenge of the immunized animal failed to grow demonstrating that the tumor had expressed antigens distinct from the host tissue (3). Thus, it has been relatively easy to demonstrate strong antigenic changes following neoplastic transformation by cancer inducing agents such as viral or chemical carcinogens. Spontaneous tumors appear to be less immunogenic than deliberately induced tumors. However, there are now reports of the presence of tumor associated antigens on spontaneous tumors. For example, a monoclonal antibody (B72.3) generated to membrane preparations of a mammary adenocarcinoma metastasis binds to 50% of breast carcinomas and to 80% of colon carcinomas, but not significantly to normal adult tissues tested (4, 5, 6). Recent evidence suggests the antibody detects a mucin like molecule (7). Inappropriately expressed blood group antigens are found on some tumors . For example there are suggestions that blood group "A-" and "B-" like antigens are expressed on tumors in patients who are otherwise blood group " O " (8, 9, 10). Possibly the the most striking example of inappropriate blood group antigen expression in cancer tissue comes from an observation by Levine (11). A female patient with gastric

cancer showed complete tumor regression after recieving an incompatible blood transfusion. It was later discovered that the patient was "pp" blood type while the mismatched blood was "P" type. The tumor tissue, when examined, was determined to have " P" like surface antigens (11). It appears that the patient mounted an anti "P" immune response after receiving the blood transfusion and this immune response induced the regression of the tumor. The complexity of tumor immunology is demonstrated by the B16 melanoma cell line, which is non-immunogenic using standard immunological procedures but can be made immunogenic by treatment with bromodeoxyuridine (BDU). The BDU treated cells can be used to immunize a syngeneic host against the growth of the parent B16 line, indicating the presence of antigens which can be recognized by the immune system (12). These demonstrations of the existence of tumor associated antigens and tumor immunity provide the rationale for tumor immunology and hope for tumor immunotherapy.

Tumor associated antigens can be characterized by at least two sub classes : 1) tumor specific, thus not expressed in any other tissue, or 2) abnormally expressed, either qualitatively or quantitively, in tumor tissue and thus functionally tumor associated. Examples of the former class are virus or chemically induced antigens and, of the latter, oncofetal or developmental antigens.

Tumor Associated Carbohydrate Antigens

Oncogenic transformation of cells is often accompanied by dramatic changes in the composition and architecture of cell-surface glycolipids and glycoproteins (13), including the secreted glycoproteins constituting the mucins produced by many tumors. These changes are in many cases sufficiently different from the progenitor cells to be recognized by the body's immune system. The exact significance of such changes is not totally understood but it has been suggested that the aberrant carbohydrate expression may reflect developmental arrest of the cells (14). The aberrant surface carbohydrate expression may be intimately related to the invasive and infiltrative properties of the tumor cells and provide the key to the failure of the tumor cell to express normal "functional cell contact" and "cell communication" (15, 16).

Aberrant surface glycolipid expression can be the result of (17, 18): a)blocked synthesis which is sometimes accompanied by the accumulation of precursor oligosaccharides, b)loss of crypticity at the cell surface, or c) the activation of glycosyltransferases in the progenitor cells resulting in carbohydrate neosynthesis. Glycoprotein changes in tumors may include a) the presence of large molecular-weight O- and N-linked oligosaccharides (13,19,20,21,22) b) the appearance of densely o-glycosylated structures (23,24), and c) the presence of either incomplete or neosynthesized O-glycosidic-linked structures (17). The structural changes in the carbohydrate content, particularly glycolipid

content, of the tumor cell membrane may also result in an altered fluidity of the membrane which in turn may affect the function of receptors, membrane enzymes, and the over all cellular metabolic turnover. Such changes in surface structure may contribute to the failure of these cells to respond normally to their environment.

The Thomsen-Friedenreich Cryptic (TF) Antigen

The original report on the TF antigen had no relationship to tumor immunology. It was observed that if red blood cells were left at room temperature for some time they became panagglutinable by human sera (25). This phenomenon was later ascribed to bacterial or viral contamination which carried with it neuraminidase (NE) activity and thus revealed the normally cryptic TF-antigen, against which there are high titres of a naturally occurring antibody in all normal human sera (26). The TF determinant can be revealed on red blood cells by treatment with NE (27). The TF antigenic determinants are described as part of the MN blood group system present on glycophorin in the red blood cell membrane. Biochemical work reveals the TF antigenic determinant as B-Gal-(1-3)-a-GalNAc which is attached to the glycophorin molecule through an O-serine and/or O-threonine linkage (28). The Tn structure is the immediate precursor of the TF antigen, and is reported to be a-GalNAc-O-Ser/Thr (28). Normally the TF determinant is cryptic due to the presence of a terminal sialic acid residue, while the Tn determinant is exposed only in individuals with a recessive genetic dissorder (29)

Springer (30) claims that the TF and the Tn antigens are exposed in over 90% of breast, lung and pancreatic cancers. He also reports increased immune activity , both humoral and cell mediated, towards these antigens by most cancer patients. These claims are based on studies using crude red blood cell membrane preparations as antigens by which means it is impossible to determine the fine specificity of the response (30). Other clinical investigators report a decrease in the levels of natural anti-TF antibodies in patients with advanced disease suggesting that the tumor may express TF-antigen which complexes with the antibody and decreases the concentration of free antibody (31). In some tumor models the expression of TF antigen by the tumor correlates with the tumor aggressiveness. Certain human cancers such as malignant urinary bladder carcinoma also express TF and Tn antigens in a pattern that correlates with the aggressiveness of the tumor, and may therefore have some prognostic value. (32,33).

Early in the history of tumor immunology it was recognized that altered carbohydrates may be immunogenic (34). Additional evidence that altered tumor associated carbohydrates are potentially immunogenic comes from experiments where animals were immunized with liposome incorporated glycolipids extracted from SV40- transformed cells. The immunized animals were shown to be resistant to tumor growth following a challenge with an injection of live tumor (35) suggesting that the glycolipid carried the immunogenic determinant. From clinical observations it is known that serum from patients with ovarian germ cell tumors contains antibodies which react with F9 cells and glycopeptides isolated from F9

cells, while normal sera lack this activity (36). An alternative approach resulted from human monoclonal antibodies produced by EBV transformation of peripheral blood B-lymphocytes from a melanoma patient (37). One monoclonal antibody OFA-1 detected an oncofetal antigen expressed by melanomas, some brain tumors and breast cancers. The antigen detected by OFA-1 was shown to be GM_2 (38). Another monoclonal antibody produced in a similar manner detected a determinant associated with neuroectodermal tumors, which is present on GD_2 (39). Again, Springer's claim that adenocarcinoma patients have both cell mediated and humoral responses to the TF and Tn antigens (40,41) also suggests that spontaneous tumors have tumor associated carbohydrate antigens.

ANTITUMOR EFFECT OF THE IMMUNE RESPONSE

Antibody mediated immunity.

Demonstrations of the presence of antigens on tumor tissue and detection of autologous tumor antigens in patients' sera do not necessarily imply that an effective anti-tumor immune response will be induced. In fact many immunogenic tumors appear to grow in the face of an ongoing anti-tumor immune response. The ineffectiveness of the immune response on the growth of an established tumor may be due to particular properties of the tumor such as weakness of tumor transplantation antigens or hiding of surface antigens as in antigen modulation (42) and production

of cell associated mucins (43). Alternatively the ineffectiveness of the immune response may originate with the class of the immune response induced. Tumor growth may promote the generation of suppressor T cells (44) and tumor induced generation of soluble blocking factors has been reported (45). That anti-tumor immune responses may affect tumor growth has been clearly shown. For example, in 1964 Moller demonstrated that tumor specific antisera, in contrast to sera specific for an irrelevant antigen, has the capacity to both enhance and inhibit tumor growth. (46). Effective humoral anti-tumor immune reactivity was demonstrated using a monoclonal antibody directed against a tumor associated glycolipid. Administration of this monoclonal antibody to an animal inoculated with L5178 lymphoma prevented the growth of the lymphoma (47).

Cell Mediated Immunity

There is a great deal of evidence from experimental systems that T cell mediated anti-tumor immunity may cause the initiation of tumor rejection. At present the exact roles of the different subclasses of the T cell mediated anti-tumor responses remain unclear. In fact the relative importance of the different subclasses may be difficult to determine, particularly in light of recent reports of lack of correlation between T cell populations mediating in vitro anti-tumor activity and in vivo tumor rejection (48).

The existence of immunosuppressive T cells has been clearly demonstrated (49,50,51,52,44), particularly in animals with large tumor

burdens. A population of cells expressing CTL activity in vitro can prov de protection against an established tumor, following treatment of the recipient with cyclophosphamide to abrogate active suppression of the rejecting response (53).

Numerous reports imply DTH activty as the effector in the rejection of some tumors (54,55,56). Perhaps the most convincing evidence of anti-tumor DTH activity is the demonstration that Lyt1+2- cells, activated with the appropriate antigen in the presence of syngeneic antigen presenting cells (APC), activated macrophages to display non-specific antitumor activity. An implantable micro growth chamber, divided into two compartments by a semi-permeable membrane, was used. Tumor specific DTH effector cells (Lyt1+2- cells) together with syngeneic APCs and specific antigen were placed in one chamber while the tumor cells and macrophages were placed in the other chamber. The loaded micro chamber was implanted into the mouse peritoneal cavity. It was later retrieved and the tumor cell growth examined. This experiment showed a requirement for specific antigen and syngeneic APCs in the T (DTH) cell chamber while the final inhibition of tumor growth was non-specific (57). A limitation of these studies was that the nature of the tumor associated antigen was not defined.

Delayed Type Hypersensitivity

Historically Koch is credited with the initial recorded observation of DTH. He observed, in the early 1900's, the dermal reactivity of

tuberculous men and animals to exposure of tuberculin (58). The DTH reaction is for this reason often refered to as the "tuberculin reaction". Nearly half a century later the first definite evidence for lymphocyte involvement came forth from Landsteiner and Chase (59). Using guinea pigs they demonstrated that DTH activity could be transfered with lymphocytes but not with immune serum from a sensitized to an unimmunized animal. It was later demonstrated that DTH is a T cell dependent phenomenon (60).

The cell surface phenotype of the majority of murine T (DTH) cells has been determined to be Lyt 1+ 2- (61) but there are also reports of Lyt 1- 2+ DTH effector cells (62). These surface markers appear to correlate with the MHC restriction requirement -that is, Lyt1+2- cells are class 2 restricted while Lyt 1- 2+ cells are class I restricted.

At the site of the antigen deposition the T(DTH) cells interact with the antigen and respond by producing 1)antigen specific factors, 2)antigen non-specific factors and 3) other lymphokines (63,64). There are reports of a 10,000 dalton transfer factor which transfers DTH activity (65), and an antigen specific DTH transfer factor has recently been reported in the murine system (66).

Lately it has become evident that two separate cells may be a required to produce a DTH reaction. The first cell (Lyt1+) is required for vasodilation and the second cell is the more conventional (Lyt1+ Lyt2-) DTH effector cell (67).

The local release of lymphokines by T (DTH) (Lyt1+2-) cells in response to the antigen attracts and activates cells mainly from the monocyte-macrophage lineage. The DTH lesions are thus characterized by

neutrophil and mononuclear cell infiltrate and gross induration, which peak at 24-48hr after antigen administration.

DTH has been demonstrated in response to many cellular antigens, soluble proteins, neoantigens created through skin painting with reactive compounds such as TNCB (trinitrochlorobenzene), to haptens such as TNP (68) and metals such as nickle and chromium (69). The evidence for carbohydrate (CHO) recognition by DTH cells (reviewed in CHO recognition section) is at best incomplete and controversial.

RECOGNITION OF CARBOHYDRATE ANTIGENS.

Non Immune Carbohydrate Recognition

Complex carbohydrate structures have the potential to encode biological information (70). A good deal of evidence indicates that carbohydrate recognition may play a role in a number of cellular interactions such as 1) cellular differentiation during development (70,71), 2) homing of cells to specific organs (72,73), 3) cell-cell adhesion (74), 4) cell-substratum adhesion (71), 5) fertilization (75), 6) contact inhibition (76) and 7) certain receptor mediated activities (72). Certain toxins, such as tetanus (78) and cholera (79,80) toxins have also been found to bind to carbohydrate determinants and may mediate the in vivo

effect through a process initiated by a carbohydrate recognition event.

Experimental models of development have demonstrated that cell adherence and aggregation are mediated by glycoproteins. For example, in the developing chick embryo a cell surface adhesion molecule (CAM), a cell surface glycoprotein, has been implicated in adhesion among cells and neurites of the developing retina and brain (71,81). Antibodies to this glycoprotein, when added to organ cultures interfere with cell-cell organization and differentiation (75). It is difficult to prove carbohydrate recognition in systems of this kind, since it may be argued that the observed interference is due to steric blocking of a protein binding site.

It has been suggested that the recognition and the removal of aged red blood cells (RBCs) by hepatocytes is mediated by the removal of the terminal sialic acid of a red blood cell glycoprotein exposing a terminal galactose determinant recognized by the hepatocyte (72). Cells treated with neuraminidase to remove the sialic acid which exposes galactose residues are eliminated from circulation at a rate many times that of normal red cell clearance, suggesting that the recognition process may involve the revealed carbohydrate determinant.

There are numerous descriptions of lectins which recognize and bind to specific carbohydrate structures. The specificity of these protein carbohydrate interactions has been determined with respect to the moieties recognised on the carbohydrate molecule. Studies using lectin IV of Griffonia simplicifolia suggest, that at least for some lectins, hydroxyl groups on the carbohydrate cluster to form a surface for polar interaction with the lectin.

Within the immune system there are multiple examples of carbohydrate (glycoprotein) involvement in biological recognition. Apart from those involving lymphocyte antigen recognition, which will be discussed separately, these include such phenomena as 1) the homing of lymphocytes to lymph nodes (82), 2) the activation of complement (83), 3) NK cell target recognition (84,85), 4) T cell sheep RBC rosettes (86) and 5) lectin and mitogen activation of lymphocytes (87).

Lymphocyte homing and recirculation is poorly understood, but is likely to involve some type of organ- or tissue- specific receptor (89). Treatment of thymocytes with neuraminidase (90) or lectins (91) alters their pattern of recirculation and distribution after injection, suggesting carbohydrate involvement. However, it is difficult to draw conclusions from such experiments since the altered cell surface may cause the cells to aggregate and clump and thus cause differences in distribution. Studies by Stoolman and Rosen (92) indicate that lymphocytes bind to glycoconjugates at the high endothelial venule in homing. The demonstration that this attachment can be inhibited by specific sugars, provides more convincing evidence for carbohydrate involvement in lymphocyte recognition.

The significance of carbohydrates in the complement-antibody interaction was shown by Wigzell (93). A clone producing IgG monoclonal antibody of subclass 2b was grown in the presence of tunicamycin ,which interferes with the formation of asparagine-linked carbohydrate chains. The resulting monoclonal antibody depleted of carbohydrate retained its antigen binding specificity and protein A reactivity, but lost its ability

to activate complement . The ability to participate in antibody dependent cell mediated cytotoxicity (ADCC) and to bind to macrophage Fc receptors was also lost in the carbohydrate depleted antibody. These findings suggest that recognition of carbohydrate determinants is important in the activation of complement and in the Ab-Fc receptor interaction.

T cells are known to form rosettes with allo- and xeno- geneic red blood cells. For example, human T cells will bind to the surface of sheep red blood cells and form rosettes (86). This binding can be enhanced by treating either cell population with neuraminidase (94), and inhibited by specific glycopeptides, suggesting that the binding is mediated via a glycoconjugate and a complementary receptor.

Certain lymphokines such as macrophage migration inhibition factor (MIF),macrophage activation factor (MAF), and lymphocyte migration inhibition factor (LIF) and monokines such as interferon can be inhibited by monosaccharides suggesting the involvement of carbohydrate determinants in the receptor recognition (95,96,97). The in vivo activation of lymphocytes by plant lectins and mitogens is also dependent on certain cell surface carbohydrate moieties (98), as is the adherence of bacteria to animal cells. Bacterial adherence to human lymphocytes can be inhibited by some monosaccharides and lectins. For example, bacterial binding to macrophages or monocytes is inhibited by the addition of mannose (99) suggesting that the recognition is at least in part carbohydrate mediated.

CARBOHYDRATES AS ANTIGENS

Specific recognition of Carbohydrate determinants by antibodies

There are many reports of humoral immune responses towards carbohydrates. In 1923 Heidelberger and Avery discovered that polysacchrides were antigenic and were able to conduct quantitative studies of antibody-antigen complexes (100). Other early studies of carbohydrate antigens were of the blood group antigens, which proved to be glycolipid in nature (101). Normal individuals have antibody directed towards their non-expressed blood group antigens including ABO, Ii, Le, M, N, and P structures. These antibodies are probably produced in response to cross reacting antigens of the intestinal flora. (102). Certain myeloma proteins and monoclonal antibodies (103) can be specifically inhibited by defined carbohydrate structures suggesting the involvement of carbohydrate in the antigenic determinant detected by the antibody. The fine specificity of monoclonal antibodies and of polyclonal anti-sera and their recognition of carbohydrate antigens has been determined through inhibition studies with synthetic carbohydrate antigens and their analogs. A monoclonal antibody made against neuraminidase treated red blood cells was specificially inhibited by synthetic B Gal (1-3) GalNAc, thus demonstrating specificity for the TF hapten (104). Inhibition studies using specifically modified analogs of carbohydrate antigens suggest that modifications which interfere with the formation of certain intramolecular

hydrogen bonds abrogates the ability of the analog to inhibit the binding of a monoclonal antibody or a lectin to its specific carbohydrate antigen. These studies indicate that the molecular interactions between the carbohydrate antigen and the antibody occurs such that hydrophobic bonding is favored and certain intramolecular hydrogen bonding in the carbohydrate moiety may be critical in order for the antibody to bind to the carbohydrate antigen. (105).

Recognition of carbohydrate antigens by T cells

The role of carbohydrate determinants in T cell responses has remained elusive despite efforts by a number of investigators to uncover it. Carbohydrate determinants in T cell mediated responses have been studied as 1) part of the major histocompatibility complex (MHC) antigens and 2) as determinants on nominal antigens seen in the context of the MHC antigens. The two basic approaches in the attempt to determine the role of CHO in cell mediated immune responses have been to 1) alter the antigen by either removing or inhibiting the attachment of the carbohydrate to the glycoprotein and 2) attempt to inhibit the T cell recognition of its antigen by the addition of free carbohydrate. Both of these approaches have given variable results.

The CTL killing assay has been most frequently used to determine the effects of the experimental modifications of cell surface carbohydrates. Cytotoxic lymphocytes respond to specific antigen in the context of MHC

gene products (106). Both MHC and nominal antigens may be glycosylated (107), such that alteration of cell surface glycosylation may effect either or both of these molecules. Efforts to determine the effect of inhibiting glycosylation of cell surface molecules have resulted in conflicting reports. Alteration of CTL target cell surface carbohydrates by treatment with glycosidases or pretreatment with glycosyl- transferase inhibitor during cell glycoprotein synthesis have 1) stimulated (108,109,110), 2) inhibited target recognition (111,112) or 3) had no effect on recognition by CTL (113,114). Similarly, alterations of cell surface carbohydrates have had variable effects on their ability to stimulate in vitro T cell cultures. Goldstein (115) finds no difference in CTL stimulation after removal of the cell surface carbohydrate, while others have found that removal of carbohydrates 1) increases (109) or 2) decreases (116) the ability of the cell to stimulate a mixed lymphocyte reaction.

The evidence that carbohydrate determinants may play a role in cell mediated immune recognition is inconclusive and conflicting. It is difficult to determine if the carbohydrates are recognized directly as immunogenic determinants or if their effects are indirect. Alteration of cell surface glycoproteins is likely to have a wider effect than simply removing an immunogenic determinant (for example it may affect membrane and protein stability), and inhibition of glycosylation may interfere with the integration of cell surface molecules into the cell surface membrane (117). It is conceivable that the removal of carbohydrate (or the inhibition of its attachment) to a nominal antigen may interfere with the

association of that antigen with the MHC gene product. In a CTL assay the removal of the proposed carbohydrate determinant recognized by the T cell cannot be differentiated from the inhibition of nominal antigen to associate with the MHC restricting molecule. Difficulty in interpreting these results have led to other experimental approaches.

For example, it has been attempted to inhibit target recognition by CTL using glycopeptides and oligo- and mono-saccharides. Glycoconjugates isolated from cell membranes can inhibit CTL target cell recognition in an H-2 specific manner (118). Mono- and oligo- saccharides can block in vitro mixed lymphocyte reactions (119,120). These inhibition experiments provide more convincing evidence for T cell recognition of a specific determinant that is at least in part carbohydrate in nature.

The capacity of carbohydrates to induce and/or trigger a Delayed Type Hypersensitivty (DTH) T cell reaction has received little attention. Klein states " DTH can be induced by a wide variety of natural and synthetic proteins; firm evidence for DTH induction by other substances is not available. Claims of DTH elicited by polysaccharide injection have been made, but in none of these experiments has the presence of contaminating proteins been rigorously excluded, and whenever a pure polysaccharide was used, no DTH could be obtained." (121). It is possible that there exist different requirements with respect to the antigen structure for the induction of the DTH cell population and the triggering of the final effector function, such that an anti-CHO DTH reaction can be induced only if the CHO serves as a hapten on a protein carrier.

It may be argued that carbohydrate determinants may be recognized by

the T cell suppressor sub-population. Evidence for this argument comes from experiments in which it was demonstrated that the determinant on Mycobacterium leprae to which suppression may be induced, represents a glycolipid (122).

Evidence from Parish and collegues suggests that there are two kinds of Ia determinants, one due to the intact proteins and the other to the carbohydrate moieties (123,124). Speculation on the functional role of these two forms of the Ia gene product suggest that the Ia region may code for a glycosyl transferase which itself carries antigenic determinants but interacts with other cells of the immune system via the recognition of its appropriate carbohydrate substrate. If true this form of self recognition may constitute another form of T cell carbohydrate interaction, differing drastically in nature from the recognition of carbohydrates as antigens. If recognition of self, for example Ia, involves recognition of carbohydrate structures, then data from carbohydrate MLR type studies may reflect alterations of this type of interaction rather than T cell antigen recognition.

In summary, carbohydrate determinants on cell surface glycoproteins or glycolipids play a role in biological recognition such as cellular recognition during development and homing. Within the immune system, carbohydrate recognition is also evident. It appears to be important in the activation of complement, macrophage binding and in some factor mediated processes such as MAF binding. Carbohydrate determinants are also responsible for the specificty of some immune responses. This has been clearly demonstrated for humoral responses. It has proved to be difficult

to clearly demonstrate carbohydrate specificity in cell mediated responses. The evidence for carbohydrate specificity in cell mediated immune responses has been conflicting and inconclusive.

A MODEL FOR IMMUNE RESPONSES TO CARCINOMA ASSOCIATED CARBOHYDRATE ANTIGENS-OF MAN.

The TA3 tumor model was developed from a spontaneous murine mammary adenocarcinoma from the A/J mouse strain (125). It has since spontaneously given rise to a subline, TA3Ha, which is non-specific with respect to the strain of mice in which it will grow, while the parent line TA3St grows only in mice with the H-2a haplotype (126). The most obvious difference between these two sublines is the glycocalix of the TA3Ha line which is absent in the TA3St line. This glycocalix is primarily composed of the glycoprotein epiglycanin (127), which contains multiple TF and Tn antigenic determinants (128). Epiglycanin (epi) is a glycoprotein, with a m.w. of approximately 500,000 and 75-80 % of the molecule consists of carbohydrate (128,129) Epi resembles many human tumor-associated mucin-like molecules recently described (21,130,131). Codington and co-workers (131) recently showed that epi cross-reactive antigens are found in the peritoneal or pleural fluid and in the sera of patients with metastatic cancer but not in normal sera. TA3 is one of the few cell lines for which a cell surface mucin is defined, and which lends itself to a

systematic analysis of the immune response against defined carbohydrate determinants and its affect on tumor growth.

Specific metastatic variants, which show organ specific metastasis to liver or lung have been selected from the parent lines . These metastatic variants retain "TF-like" antigen expression in the metastatic foci (132,133). This is similar to the clinical situation where it has been reported that carcinoma metastases retain "TF-like" antigen expression (17). Due to these features the TA3 model is also ideally suited for analysis of the importance of the immune reponse against metastatic spread.

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Chapter 2

Generation of DTH Effector Cells Against a Tumor-Associated Glycoprotein (TAG) and its Synthetic Epitopes

INTRODUCTION

The ability of T-cells to recognize carbohydrate haptens has been a long debated issue. In attempts to resolve the controversy experimental evidence has been put forth to suggest that alteration of the carbohydrate portion of an antigen 1) inhibits (1,2), 2)stimulates (3,4,5) or 3) has no effect (6,7) on the ability of T cells to recognize and respond to the antigen. The difficulties experienced in the T-cell system may be due to the well known phenomenon of self MHC restriction of T-cell responses (8). Thus, since antigen is generally seen in the context of self, attempts to determine T cell recognition specificity may be complicated by the requirement for antigen processing and presentation.

We have approached the question of T-cell recognition of carbohydrate antigens by using a tumor associated glycoprotein, epiglycanin (epi) and synthetic carbohydrate haptens which are identical to the known determinants on the epi molecule. Epi is produced by a murine spontaneous mammary adenocarcinoma cell line, TA3Ha (9). It is 75-80% carbohydrate, and carries the immunodominant Thomson-Friedenreich (TF, B-Gal-(1-3)-a-GalNAc-) determinant and its immediate precursor Tn (a-GalNAc-O-Ser) antigen (10). The TF determinant is cryptic in normal human red blood cells but can be revealed by neuraminidase treatment,

which removes the terminal NANA unit (11). The TF, Tn and related haptens were synthesized, conjugated to a carrier molecule (HSA or KLH) and used in these experiments to attempt to determine whether murine T-cells can recognize and respond to carbohydrate haptens. Here we show that the T (DTH) cells primed to epi, or the appropriate s-TAG can be elicited in a local DTH reaction by epi and synthetic haptens linked to HSA but not by the carrier alone nor by an inappropriate sugar (eg. fucose) linked to the carrier. The DTH response can also be induced in vivo by irradiated TA3Ha cells and elicited by neuraminidase treated red blood cells, but not by normal untreated red blood cells. It is well established that the normally cryptic TF antigen is revealed by neuraminidase treatment of human red blood cells (11). The in vivo experiments are consistent with the observed specificity of the in vitro primed cells and show that it is possible to generate DTH effector cells with specificity determined by the carbohydrate portion of the molecule. These findings show that the DTH primed cell population has specificity, at least in part, for the carbohydrate hapten.

Materials and Methods

GENERATION of DTH EFFECTOR CELLS

A modification of the culture conditions described by Bretscher (31) was used to generate antigen specific DTH effector cell populations. CAF_1/J (Balb xA/J) mice from the local animal unit were immunized with 30ug Epi in 50% CFA ip (or 2 ug of any of the synthetic antigens hereafter refered to as s-TAGs; synthetic tumor associated glycoconjugates), and boosted one week later with a similar injection. Seven to ten days after the last injection the animals were killed , their spleens removed and passed through a metal mesh to make a single cell suspension. The cells were washed and plated in costar wells at 1.5 x 10^7 cells / 2ml medium (RPMI + penicillin + streptomycin (10 units/ml) + 10% fetal calf serum) / well. Each well was pulsed with antigen, 10ug epi or 1-2ug of any of the synthetic antigens, for 2 hours at 37 degrees C. in 5% CO_2 in air after which 6 ml of medium were added to each well. The cells were cultured at 37 degrees C. in 5% CO_2 in air for 6 days and then harvested by gently dissociating the cells from the plastic with a rubber policeman. The cells were washed in PBS (4 x 10ml). The primed cells were tested for DTH activity in a local DTH footpad assay.

ANTIBODY TREATMENT of EFFECTOR CELLS

A primed cell population was obtained as described above ,washed, counted and distributed to separate tubes for the antibody treatment. The cells

were gently pelleted and resuspended in Leibowitz (Leib) medium containing (1) anti-Thy1.2 antibody (at 1/1000 diln., obtained from New England Nuclear, NEN), (2) anti-Lyt1.2 antibody (at 1/1000 diln.,obtained from NEN) or (3) anti-Lyt2.2 antibody (at 1/1000 diln., obtained from NEN.) at 2 x 10^7 cells / ml, and incubated at 4 degrees C. for 45 minutes. The cells were then pelleted, resuspended in guinea pig complement (1/10 diln. in Leibovitz medium, obtained from M.A. Bioproducts.) at a density of 2 x 10^7 cells/ ml and incubated for 30 minutes in a 37 degree centigrade water bath. There after the cells were washed with Leibowitz media + 10% FCS (4 x 10ml), counted and tested for DTH activity.

LOCAL DTH ASSAY

A modification of the DTH assay described by Bretscher (31) was used. In short, cells harvested from six day cultures were washed with PBS (4 x 10ml) and counted. The primed cells were aliquoted and pelleted, then resuspended in PBS containing antigen (2mg/ml, 0.5mg synthetic antigen / ml, 100-200mg s-TAG/ml of microsphere suspention, appropriate volumne of 20% NERBC or 4 x 10^7 irradiated (10,000 rads) TA3Ha cells /ml) at a concentration of 4 x 10^8 primed cells / ml. The mixture was incubated at 37 degrees C. for 30 minutes, and 25 ul of the mixture were injected into the right hind foot pad of an unimmunized syngeneic mouse. The controls were separately injected with primed cells or antigen. At 24 and 48 hr after the injection , the thickness of the right and the left hind foot pads were measured with an oditest (0-10mm,0.01mm) thickness guage

(obtained from H.C Kroplin, W.Germany) and the DTH swelling estimated.

Estimate of DTH:

=[swelling of injected foot] - [swelling of uninjected foot]

Net DTH:

=[Average DTH (primed cells +antigen)]

- [Average DTH (primed cells only)]

One unit of swelling is 10^{-2} mm.

SYSTEMIC DTH ASSAY

The local DTH assay was modified to a systemic DTH assay. 5×10^7 primed cells (in .5ml of PBS) were adoptively transfered to non-immunized syngeneic hosts via an iv transfer. The animals were then challenged with 10^6 irradiated TA3Ha cells in 25ul PBS in a local foot pad injection at various times after the iv transfer. DTH was estimated as for the local DTH assay, and net DTH calculated as the difference between DTH swelling of iv transfered mice and the DTH swelling of controls (DTH challenged normal mice).

EXTRACTION of EPIGLYCANIN

Epiglycanin (epi) was extracted from the ascites fluid of TA3Ha tumor bearing mice.

Ascites from outbred mice bearing TA3Ha ip tumors was collected, cells were removed by centrifugation, and ascites stored frozen. Before extraction, the ascites was thawed, incubated at 37 degrees centigrade for 2 hours and any aggregates were removed by centrifugation. The freeze-thaw procedure was repeated twice before PNA extraction. In some cases the ascites was also dialysed over night before extraction.

A 25-40 ml packed volume of PNA agarose (E.Y. Laboratories) were washed with PBS, mixed with ascites and tumbled gently at 4 degrees C. for 24-48 hours. The slurry was poured into a column and extensively washed with PBS. The PNA bound Epi was eluted with 100ml 0.5 M galactose.

The eluate was concentrated to 25-30 ml, dialysed against 2 x 4 liters of PBS at 4 degrees C. with continous stirring for 24 hours. The sample was lyophilized and stored at -20 degrees C in a dessicator.
The epi preparation was characterized by FPLC chromotography and the epi activity was detected with monoclonal antibody 49H.8 in an elisa assay (24). No major contaminating peaks were detected in the FPLC profile.

IN VIVO IMMUNIZATION.

Locally bred (Balb x A/J) F_1 mice were injected with irradiated (10,000rad) TA3Ha emulsified in CFA (50%) via an ip. injection. After 7-10

days the animals were killed, the spleens removed, and a single cell suspension was made by passing the organ through a metal mesh. Primed cells (10^7) were mixed with the indicated antigen and injected into the hind foot pad of a non-immunized mouse. Footpad swelling was measured at 24 hours and calculated as described above.

TUMOR CELL LINES.

The TA3Ha cell line (38) was kindly provided by Dr J.F.Codington (Mass. General Hospital, Boston MA.) and grown in vivo by weekly passage (ip) in (Balb/c x A/J) F^1 mice. Each passage-line was terminated at passage 10 and a fresh frozed aliquot started.

mKSA tumor cells (39) were obtained form Dr. P A Bretscher and grown similarly to the TA3Ha cells.

NEURAMINIDASE-TREATMENT OF HUMAN RED BLOOD CELLS.

Human red blood cells were treated with neuraminidase as described by Rahman and Longenecker (24).

HISTOLOGY.

Tissue sections of a local inflammatory site or tumor site were taken and immediatly frozen in liquid nitrogen. The frozen sections were cut and

stained with Hematoxylin and Eosin stains using standard histology methods.

SYNTHETIC ANTIGENS.

Synthetic tumor associated glycoconjugates (sTAGs), in particular TF(a) (B-Gal-(1-3)-a-GalNAc), TF(B) (B-Gal-(1-3)-a-GalNAc), and Tn (a-GalNAc-O-Ser) were obtained from Biomira, Inc., Edmonton, Alberta, Canada. See table 1 for structures and nomenclature.

MHC RESTRICTION OF DTH ELICITATION

Intraperitoneal macrophages were obtained from non-primed mice by washing of the peritoneal cavity twice with 3ml of PBS. The cells were pelleted , counted and incubated with s-TAG antigen or HSA (100ug/ ml PBS) for 2 hours at 2-4 x 10^6 cells/ml at 37 degree C. The cells were then washed four times with PBS prior to the DTH assay. One million antigen pulsed macrophages plus 10^7 primed cells were injected into the footpad of non-immunized mice, syngeneic to the primed cell population. DTH was measured at 24 hours after the injection.

ANTIGEN COUPLING TO MICROSPHERES.

Latex spheres (from Polysciences Inc., USA)were activated in accordance with the accompanying instructions and linked to 100-200ug of antigen per 100 ul of latex suspention. Uncoupled sites were blocked with NMS. The spheres were washed with PBS + 5% FCS and stored with (10mM) azide added at 4 degrees C.

RESULTS

Cell Specificity of the in vivo DTH Response

The initial experiments to determine the specificity of the anti epi response were done in vivo. Mice were immunized with irradiated TA3Ha cells (figure 1) and 10 days later the animals were killed and the spleens removed. Ten million primed spleen cells plus indicated antigens were injected subcutaneously into the footpads of normal unimmunized mice. Figure 1-1 shows that the response was elicited by irradiated TA3Ha cells, NE -RBCs, but not normal RBCs or by irradiated L1210 cells, a tumor which does not express cell surface TF antigen (24).

In vitro generation of DTH effector cells is antigen dependent

To test the antigen dependency of the in vitro generation of the anti epi DTH effector cell population, spleen cells from epi primed and unprimed animals were cultured in the presence or absence of antigen and then tested for DTH activity in a local DTH foot pad assay. Figure 1-2 shows that a DTH active cell population was induced only when the spleen cells were primed in vivo and antigen was present during the in vitro culture.

Characterization of the in vitro generated DTH response

The kinetics of the local DTH reaction was determined following the

local deposition of primed spleen cells plus epi, and DTH was measured at various time intervals after the injection. Figure 1-3 shows the absence of an early 6-10 hour swelling followed by a peak response at 24-48 hours when primed cells and antigen were injected together, while no swelling was observed when either was injected alone. The swelling decreased to an undetectable level by 96 hours (data not shown).

Cell Surface Phenotype of the in vitro Generated DTH Effector Cells

In order to further characterize the DTH effector cell population, the cell surface phenotype was determined using anti-Thy1.2, anti-Lyt1.2 or anti-Lyt2.2 monoclonal antibody and complement treatment prior to transfer to the footpad. Cells primed in vitro to a) epi (fig 1-4a) or b) TF(a) plus TF(B) (fig 1-4b) were treated with antibody and complement immediately prior to the DTH assay. The DTH response to both antigens was markedly reduced following treatment with either anti Thy1.2 or anti Lyt1.2 in the presence of complement but not by anti Lyt2.2 plus complement nor by complement alone (figure 1-4a,1-4b). All treatments involving both antibody and complement resulted in reduced cell numbers (see figures 1-4a and 1-4b). The cell surface phenotype of the DTH effector cell is thus Thy1.2+, Lyt1.2+ and Lyt2.2-.

Histology of local DTH site

The histology of the local DTH site was determined at 24 hours following local deposition of primed cells plus epi. Figure 1-5 shows a

massive mononuclear infiltration only when (a) primed cells and antigen were injected together. There was only a minimal infiltrate seen the the controls, which were injected with (b) primed cells alone or (c) antigen only.

Tumor Specificity of the in vitro generated DTH effector cells

In order to determine the tumor specificity of the local DTH reaction irradiated TA3Ha or mKSA, an SV40 transformed fibroblast tumor line, were used to trigger the local DTH reaction. Anti-epi primed DTH effector cells were injected together with irradiated TA3Ha or irradiated mKSA cells in a foot pad DTH assay. Figure 1-6 shows that the anti-epi DTH response was triggered by irradiated TA3Ha but not by mKSA cells.

Fine Antigen Specificity of the in vitro generated DTH effector cells

To determine the fine specificity of the DTH effector cells, purified Epi or s-TAGs (Tn, TF(a), TF(B)) or fucose linked to HSA were immobilized on sepharose microspheres (m). Primed cells were injected in a local foot pad together with the indicated antigens. The epi primed DTH was elicited by epi, Tn, TF(a) and TF(B) linked to HSA but not by Fuc-HSA nor by HSA alone, see figure 1-7a, nor was it elicited by Glu-BSA (data not shown). We confirmed that Epi does not cross react with HSA, at least at the antibody level, by testing anti-HSA monoclonal antibody and anti-HSA polyclonal antibodies for reactivity with epi. At no concentration tested did the anti HSA antibody react with the epi (data not shown).

Similarly, cells primed to Tn-HSA were tested for the ability to produce a local DTH reaction in response to epi and the various synthetic antigens. As shown in figure 1-7b, the anti-Tn DTH reaction was triggered by TA3Ha, Tn-, TF(a)-,TF(B)-HSA, but not by Fuc-HSA or by BSA alone. Other experiments show no response by the primed cell population to the HSA carrier molecule. Effector cell populations generated to TF(a)-and TF(B)-HSA conjugates had similar recognition specificities (see figure 1-8 for summary of specificity experiments).

These experiments were later repeated using soluble antigen (not linked to microspheres) which was preincubated with the primed cells for 30 minutes prior to foot pad injection. The results obtained with the latter method were essentially identical to those reported here.

Effect of the carrier molecule

To determine if the carrier molecule affects the recognition specificity, the TF(a) hapten was conjugated to KLH instead of HSA. Epi primed cells plus the indicated antigen were injected into the foot pad of normal mice. Figure 1-9a shows that the epi primed cell population recognized and responded equally well to both TF(a)-HSA and TF(a)-KLH. The response was not elicited by HSA alone, nor by Lewis^a-KLH. In a parallel experiment cell primed to the carrier alone were tested for antigen recognition in a local DTH assay. The data , figure 1-9b, suggests that no DTH activity was induced to HSA using the current priming conditions.

MHC restriction of the DTH reaction

In order to determine any MHC restriction in the interaction between the T (DTH) cell and the antigen presenting cell, peritoneal macrophages from Balb/c (H-2^d), C3H (H-2^k) and C57Bl/10 (H-2^b) mice were pulsed with (1) T(b)-HSA or (2) HSA alone washed and used in place of soluble antigen in the local DTH assay. Epi primed cells (Balb/c x A/J, H-2^d x H-2^a) plus antigen pulsed macrophages were injected in the foot pad and the DTH measured 24 hours later. Figures 1-10a and 1-10b show that a local DTH reaction was elicited only by Balb/c, C3H, B10.BR and F_1 antigen presenting macrophages. These macrophages share identity with the primed T-cell population at the H-2 locus, at least at the H-2 I- and K-regions. Furthermore since B10.BR macrophages, which can elicit a DTH reaction, and C57Bl/10 macrophages, which cannot elicit a DTH reaction, are identical except at the H-2 loci it appears that the restriction involves only the H-2 encoded products. The data suggests that carbohydrate specific T-cells respond to the antigen presented in the context of self MHC products.

Systemic transfer of effector cells.

A systemic transfer assay was used to determine the ability of the DTH effector cells to survive in vivo and to localize to a local site of antigen deposit. Fifty million epi primed cells were transfered via an iv injection into non-immunized syngeneic mice. DTH challenges, in the form

of irradiated TA3Ha cells, were given at various times after iv transfer. Control animals received a DTH challenge without prior iv transfer of primed cells. Figure 1-11 shows that a local DTH reaction can be elicited by an antigen challenge at 4 hours but not at 24 hours after the iv transfer of the primed cells.

DISCUSSION

It is well recognized that carbohydrates play an important role in cellular recognition and interactions (13,14,15). It appears that carbohydrate determinants may serve to guide cell migration and other interactions during development (16). There is also evidence that carbohydrates are involved in metastasis to specific organs (16,13), and that exposed carbohydrate determinants appear to mediate recognition and removal of aged red blood cells by hepatocytes (13).

Recognition of carbohydrate determinants by antibody is well documented (20,21,22), such that monoclonal antibody to various cellular antigens have been shown to have fine specificity determined by cell surface, immunodominant carbohydrate determinants (24). However, T cell recognition of carbohydrate determinants has been more difficult to demonstrate. Since most T cell responses appear to be directed at processed antigen in association with self restricting elements (8), attempts to inhibit T cell reactions with free hapten have met with limited success (25,27). Inhibition of CTL-target conjugate formation in a specific manner using a purifed cell surface carbohydrate suggest carbohydrate moieties are involved in the recognition event (28). The opposite approach, to remove the proposed carbohydrate determinant from a glycoprotein by enzymatic cleavage or inhibition of glycosylation (26,29), suffers from criticism of conformational stability. That is, if the removal of the carbohydrate affects the stability of the protein

conformation, then the loss of recognition associated with the removal of the carbohydrate may be due to loss of a protein determinant. Thus, to date the evidence for T cell recognition of carbohydrate determinants has only been suggestive.

We have used a different approach to attempt to demonstrate T (DTH) cell recognition where the specificity is determined by a carbohydrate hapten. The characteristic non-specific amplification of the specifically triggered DTH reaction provides a sensitive assay with which to analyse the antigen recognition specificity. We examined the recognition specificity of the triggering of a local DTH reaction using epi and its synthetic carbohydrate analogs as haptens conjugated to an HSA carrier molecule. Initial in vivo experiments suggested that a determinant common to TA3Ha cells and neuraminidase treated red blood cells could be detected in a DTH-like reaction. This determinant was absent from normal human red blood cells but was revealed following neuraminidase treatment. In order to further examine the fine specificity of the DTH effector cells we used an in vitro priming protocol involving a modification of the culture conditions described by Bretscher (31). Using this method we have shown that DTH induced to Epi, Tn , TF(a)- or TF(B) -HSA was triggered specifically by Tn, TF-, and TF(B) linked to HSA and by TF linked to KLH but not by the carrier molecule (HSA) alone, or by an inappropiate sugar linked to the carrier, for example fucose-HSA or Le(a)-KLH, and not by glucose adsorbed to BSA (data not shown). We observed no DTH to HSA following priming with either epi or the synthetic antigens conjugated to HSA. An example of the inability of HSA to induce a T (DTH) cell

population under the conditions used during the in vivo and in vitro priming is seen in figure 1-8b where animals were deliberately immunized with HSA at a concentration similar to the one used with the synthetic antigens. Tested for DTH activity in a local DTH assay together with the indicated antigens, none of the indicated antigens, including HSA, were able to elicit a detectable DTH reaction (fig 1-8b). The data suggests that under the in vivo and in vitro priming conditions optimal for the induction of an anti-TAG DTH effector cell population there is no induction of an anti-HSA DTH response. It is possible that the ability of the synthetic carbohydrate antigens to induce a T (DTH) cell population under the conditions used for in vivo and in vitro priming may be related to the lipid-like linker between the carbohydrate and the HSA. A similar role of lipid in the induction of DTH has been described (30).

Chemical analysis of epi has determined that the immunodominant Tn- and TF- like determinants were exposed but that no fucose was exposed (29). We confirmed that epi did not cross react with HSA by testing anti-HSA monoclonal antibody and anti-HSA sera for reactivity with epi. At no concentration tested did the anti-HSA antibody react with epi (unpublished result). Attempts to induce an anti-fucose DTH response resulted in non-specific reactivity. A large proportion of the spleen cells cultured in the presence of fucose-HSA were blasts and caused non-specific foot pad swelling , when assayed for DTH activity, even in the absence of antigen. The ability of fucose to stimulate lymphocyte reactions non specifically, has recently been reported by Stenakova (33).

The results reported here suggest that T-(DTH) cell specificity is at least in part determined by carbohydrate determinants on the carrier molecule. A comparison of the structures of the synthetic antigens, shown in table 1-1, revealed a number of differences between the conjugates which elicited the anti epi DTH and those which did not. The Tn, TF(a) and TF(B) conjugates each contain the GalNAc- structure which is coupled to the linker arm and attached to the HSA carrier. The fucose-HSA conjugate is made with the same linker arm as the TF conjugates. Since the GalNAc molecule contains two extended side groups, which are absent in fucose, an OH (in position 6) and an NH-COOH group (in position 2), it is possible that the absence of these groups from the fucose HSA conjugate is responsible for the inability of fucose to trigger the local DTH reaction. This interpretation is consistent with the critical involvment of the NAc group in antibody and lectin recognition of the GalNAc moiety. Our data thus suggests that the determinant recognized by the T cell involves at least the GalNAc- moiety. Both the OH and the NAc group, may contribute to the recognition of GalNAc by T cells.

It can be argued that a combination of carbohydrate and protein is recognized by the specific T cell. This possibility has not been formally ruled out but seems unlikely. The cell population primed with epi was elicited by both TF-HSA and TF-KLH but not by Le(a)-KLH or by HSA alone. Futhermore, the Tn synthetic hapten is linked to a serine via an O-glycosidic likage while the TF(a) and TF(B) are both linked directly to an 8 carbon spacer arm. These differences in linkage to the carrier and the ability of the hapten linked to different carriers to elicit the DTH

response suggests that the protein is not determining the specificity recognized by the T (DTH) cells. It is however, conceivable that the protein portion is required in order for the molecule to be processed and presented for a T cell response. Studies on antigenic structure in antibody-antigen interactions suggest that a hydrophobic portion may be neccesary for the presentation of antigenic structures (37). Hypothetically, the hydrophobic region would allow the antigen to associate with the self restricting elements on the cell surface membrane. Further experiments, with T cell clones, are needed to determine if free carbohydrate can fulfill the function of the antigen or if it is neccesary to present the antigen on a protein or possibly a lipid like carrier. This question has not been addressed in this series of experiments.

The carbohydrate-specific reaction is dependent on a Thy1.2+, Lyt1.2+, Lyt2.2- cell population and reaches maximum swelling, characteristic of DTH, in 24-48 hours after local antigen challenge (34). The histology of the local site showed a massive neutrophil and monocyte infiltrate, as is characteristic of DTH reaction (12). Thus it appears that the carbohydrate specific response fulfills the criteria of a classical DTH reaction.

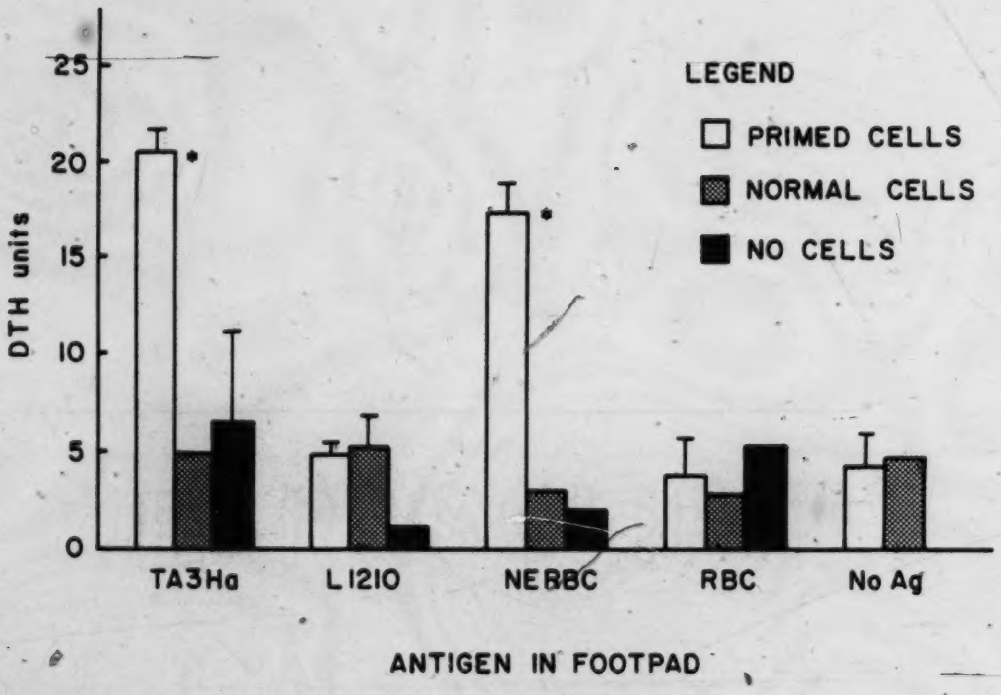
T cell responses ascribed to protein determinants are MHC restricted (34). To determine if T cell responses involving carbohydrate antigens were restricted at the T cell-macrophage interaction level, we looked for restriction in antigen presentation to elicit the final effector function. The results show that T (DTH) response to carbohydrate antigens requires identity between the eliciting macrophage and the T (DTH) cell at the H-2

locus, similar to the restriction observed in the case of protein antigens. Under combinations of primed cells and antigen pulsed macrophages where there was lack of identity at H-2 D,I and K no DTH is elicited while in combinations where there was identity at H-2 I and K a DTH reaction was seen. The data suggest that identity at the H-2 I or K regions is sufficient for the interaction to occur. Thus the elicitation of the DTH response appears to be MHC restricted. Given that the carbohydrate hapten is conjugated to a protein carrier it is possible that the observed MHC restriction is a protein related phenomenon. Experiments to determine the in vivo fate of the in vitro primed effector cells indicate that an adoptively transfered DTH effector cell population is able to localize to the antigen site and mediate a local DTH reaction. An interesting observation is the short half life of the adoptively transfered DTH activity. Figure 1-11 shows that DTH can be elicited immediately after iv transfer but is lost at 24 hours after the iv transfer. If this observation represents a suppression of the adoptively transfered TAG primed cells it becomes an important consideration in relation to tumor immunotherapy using in vitro primed DTH effector cells.

Recent reports reveal that a Thy1+ Lyt 1+ 2- subpopulation of T cells can mediate in vivo protection against tumor growth (35). This T cell population is specifically triggered by its immunizing antigen but is non-specific in its final effector function, similar to a classical DTH. We reasoned that since epi is a tumor associated glycoprotein the anti-epi DTH may be able to confer protection against the growth of the

tumor. Furthermore, the DTH induced by the synthetic carbohydrates may also have anti-tumor effects. In the subsequent paper we will show that the tumor associated glycoprotein (epi) and its synthetic analogs can induce a protective T cell population which retards the growth of the TA3Ha cells.

Figure 1-1:

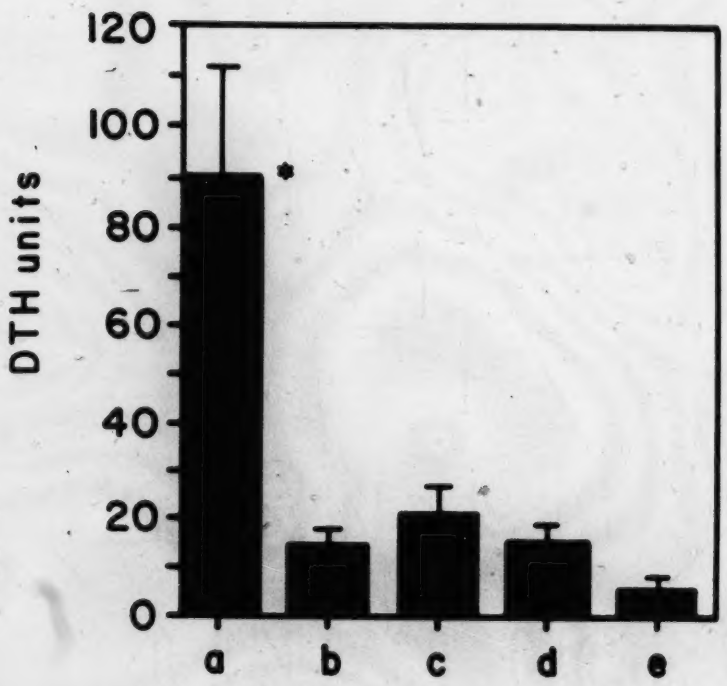


Cell Specificity of the in vivo primed DTH Response.

Spleen cells (10^7) from mice primed 10 days prior with 10^6 irradiated TA3Ha cells in were injected s.c. into the footpad of syngeneic mice together with the indicated antigen. DTH was measured 24hours after injection.

* $p < .005$ (The group injected with antigen only was used as control in the calculating the p values)

Figure 1-2:

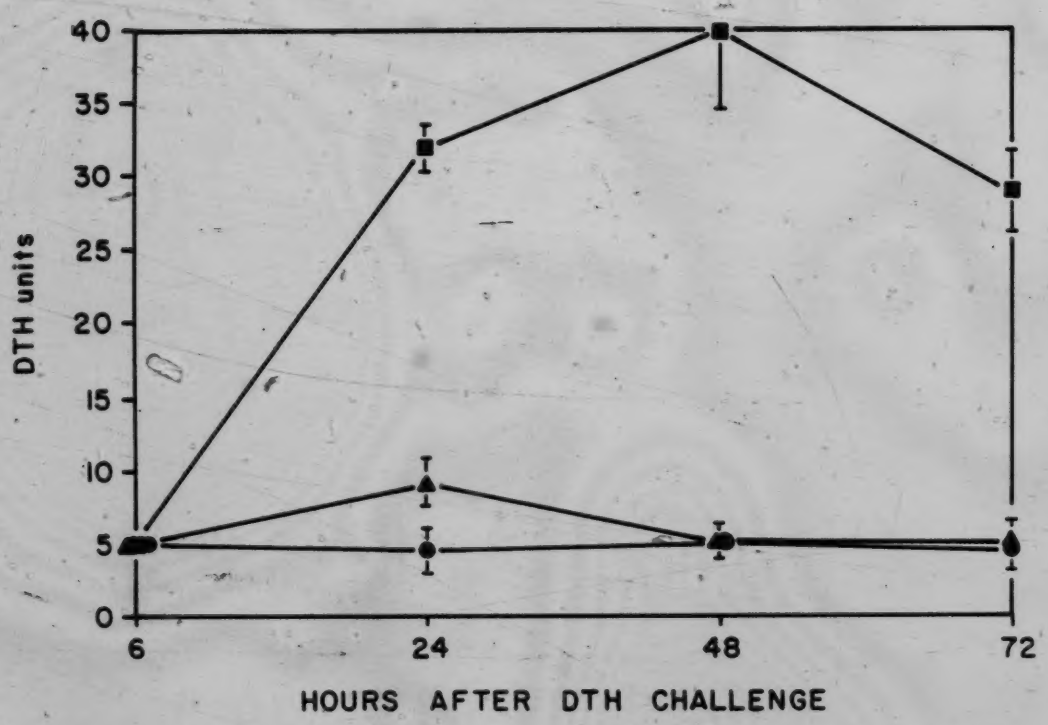


Antigen Dependency of in vitro Generated DTH Effector Cells.

Primed spleen cells were cultured (a) and (e) in the presence of antigen and (b) in the absence of antigen. Normal spleen cells were cultured (c) in the presence of antigen and (d) in the absence of antigen. The cells were harvested after 6 days in culture and tested for DTH activity in the presence of epi (a,b,c,d) and in the absence of epi (e).

* $P < .001$ (Group (e) was used as control for the calculations of the p values)

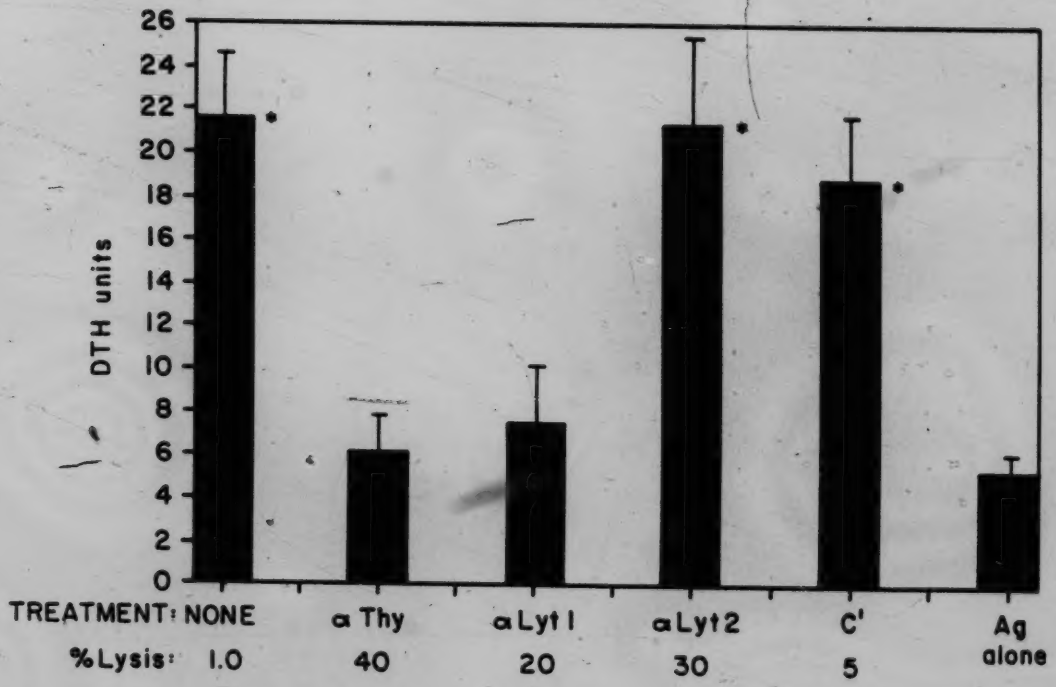
Figure 1-3:



Kinetics of the in vitro Generated DTH Response.

Ten million cells primed in vivo and in vitro with epi (■) were injected together with irradiated TA3Ha cells in a local DTH assay. Foot pad swelling was measured at various times after the injection (as indicated). Controls include either primed cells injected alone (▲) or irradiated TA3Ha cells injected alone (●).

Figure 1-4a :



Cell Surface Phenotype of in vitro Generated DTH Effector Cells.

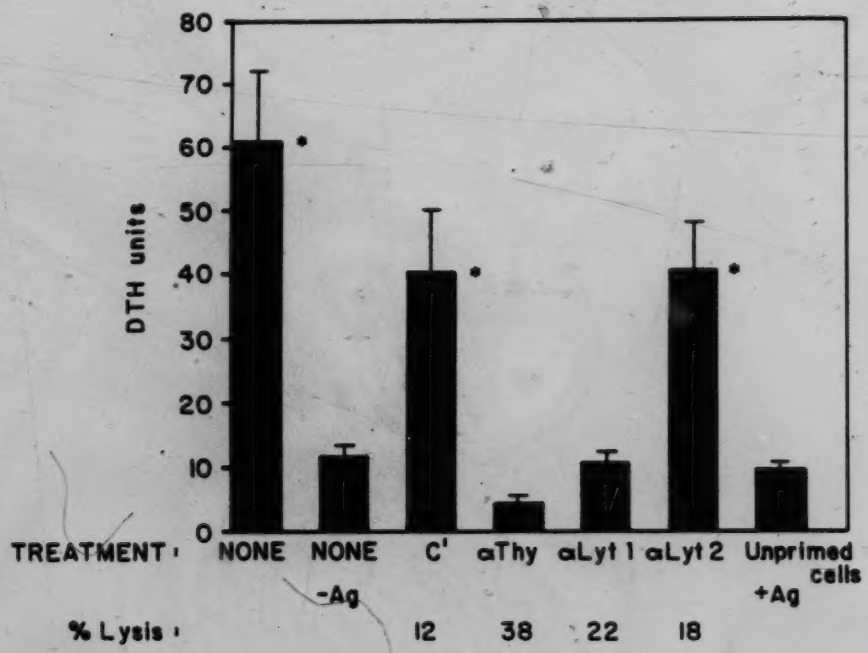
Cells primed to epi were treated with the indicated monoclonal antibody in the presence of complement immediately prior to the DTH assay. The remaining cells were injected together with epi in a local DTH foot pad assay, and DTH was measured 24 hours later.

* indicates the treatment groups which differ significantly from the group given antigen only controls, $P < .001$.

The experiment was repeated at least 3 different times using n=5 each time.

Cell treatment involving both antibody and complement resulted in reduced cell numbers (indicated in figure as % lysis).

Figure 1-4b:



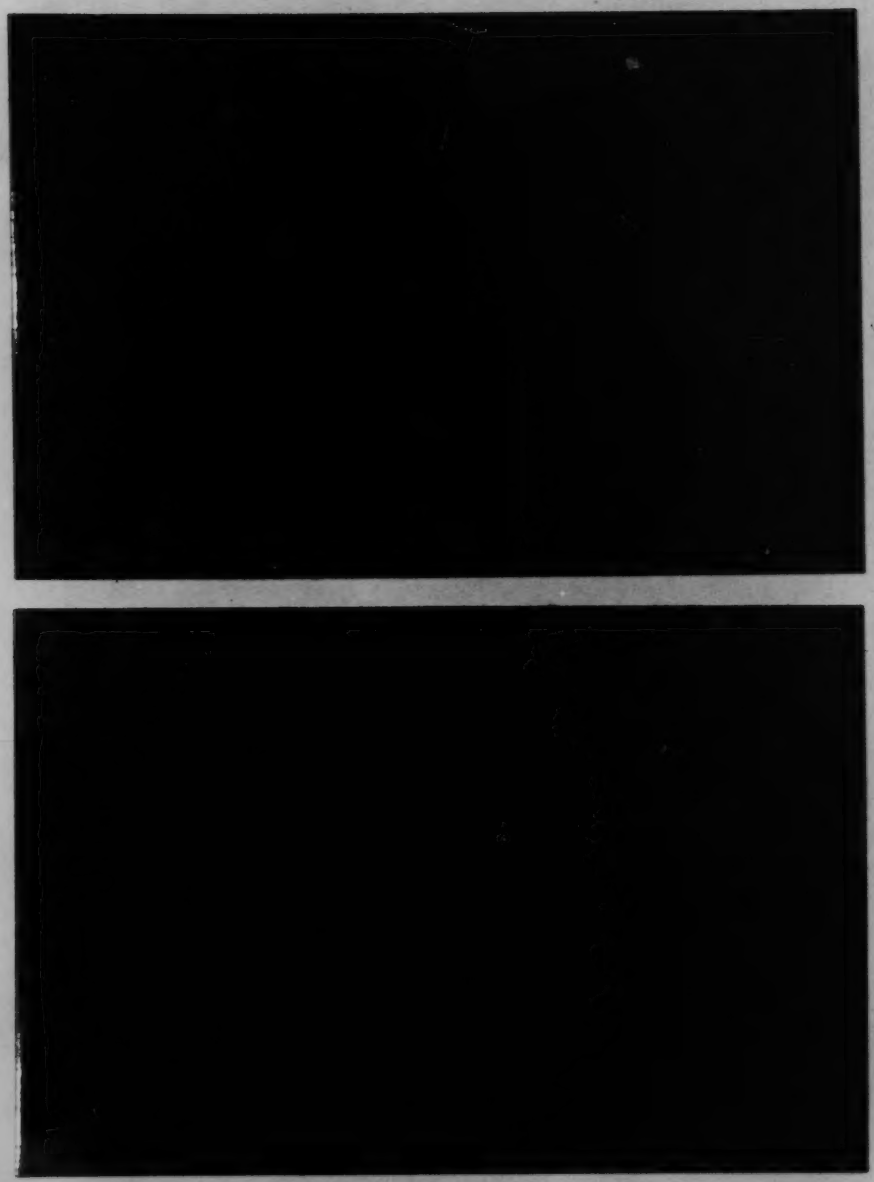
Cell Surface Phenotype of in vitro Generated DTH Effector Cells.

Cells primed to TF(b) were treated with the indicated monoclonal antibody in the presence of complement immediately prior to the DTH assay. The remaining cells were injected together with epi in a local DTH foot pad assay, and DTH was measured 24 hours later.

* indicates the treatment groups which differ significantly from the group given antigen plus unprimed spleen cells, $P < .001$

Cell treatment involving both antibody and complement resulted in reduced cell numbers (indicated in figure as % lysis).

Figure 1-5a:



Histology of the DTH site.

A frozen section of a local DTH site injected with primed cells plus antigen was made and stained with Hematoxylin-Eosin stain.

Figure 1-5 b,c :

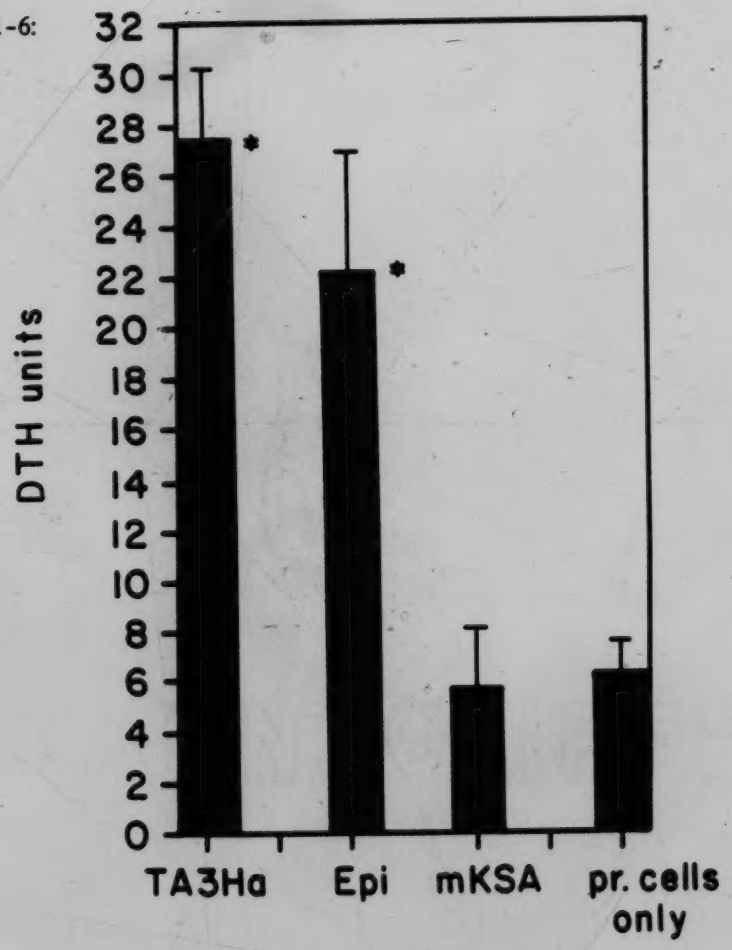
(b)

(c)

Histology controls.

Frozen sections of local sites injected with primed cells alone (b) or with antigen alone (c) were made and stained with Hematoxylin-Eosin stain.

Figure 1-6:

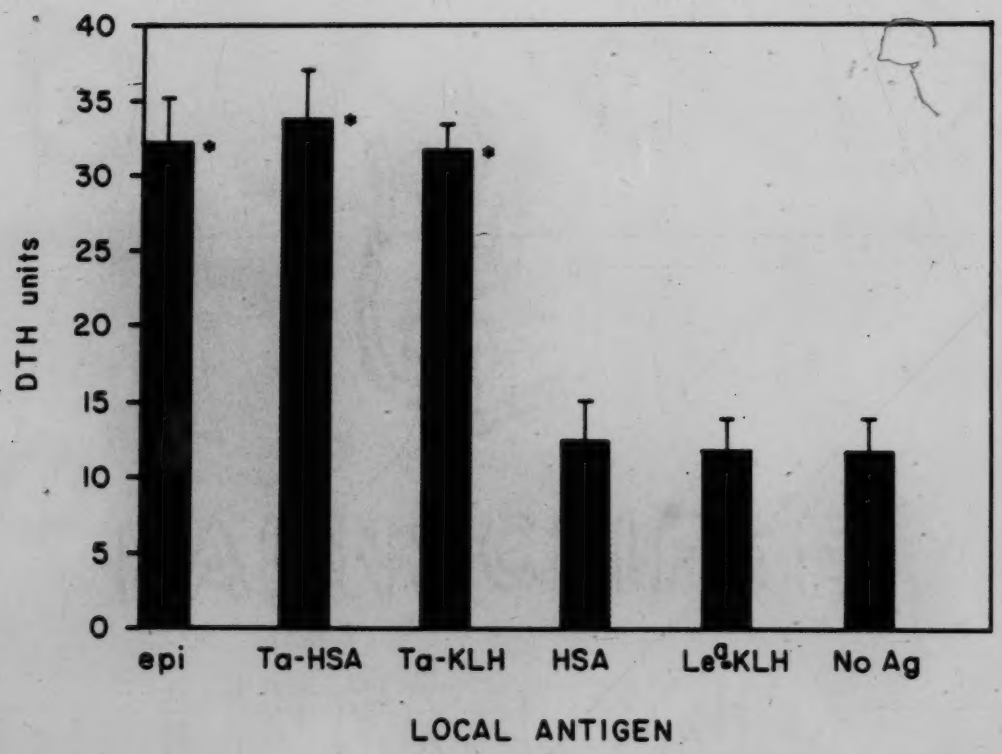


Tumor Specificity of the in vitro Generated DTH Effector Cells.

10^7 cells primed to epi in vitro were mixed with the indicated antigen and injected into the foot pad of an normal mouse. Controls were injected with primed cells or antigen separately. DTH was measured at 24 hours after the injection. Net DTH was estimated by subtracting the swelling due only to the antigen from the swelling of the primed cells injected together with the antigen.

* indicates groups which differ significantly from the swelling resulting from the primed cells alone, $P < .001$

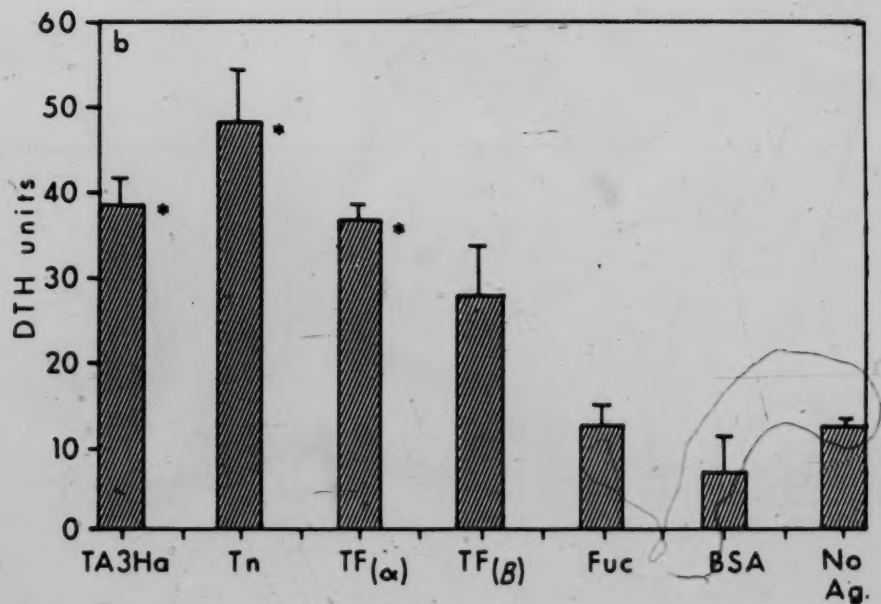
Figure 1-7a:



Fine Antigen Specificity of the in vitro Generated DTH Effector Cells. 10^7 cells primed to epi in vitro were injected together with the indicated antigens in a local DTH assay. Soluble antigens were linked to sepharose-microspheres. DTH was measured 24 hours later.

* $p < .005$ (The group injected with microspheres (M) alone was used as control in the statistical comparisons.)

Figure 1-7b:



Fine Antigen Specificity of the in vitro Generated DTH Effector Cells.

10^7 cells primed to Tn-HSA in vitro were injected together with the indicated antigens in a local DTH assay. Soluble antigens were linked to sepharose- microspheres. DTH was measured 24 hours later.

* $p < .005$ (The group injected with primed cells only was used as control in the statistical analysis.)

Figure 1-8:

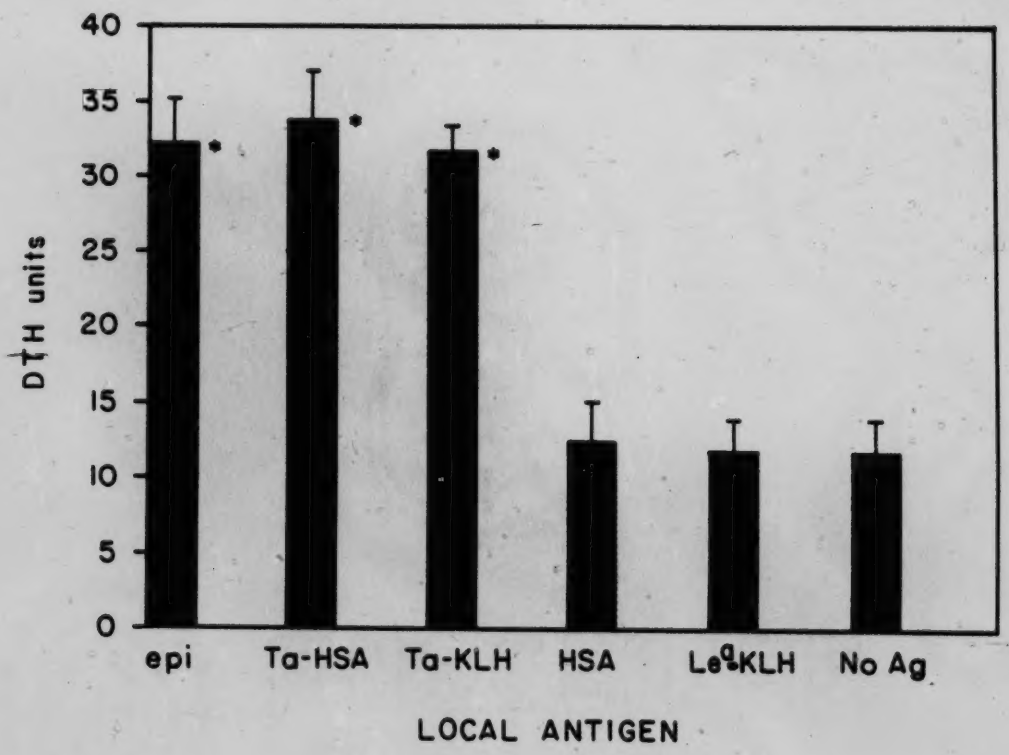
IMMUNIZING Ag:	TRIGGERING Ag:					
in vivo / in vitro	Epi	Tn	$TF_{(\alpha)}$	$TF_{(\beta)}$	Fuc	BSA/HSA
Epi:	+	+	+	+	-	-
$TF_{(\alpha)}$:	+	+	+	+	-	-
$TF_{(\beta)}$:	+	+	+	+	-	-
Tn:	+	+	+	+	-	-
None:	-	-	ND	ND	ND	ND
	TA3Ha	L1210	mKSA	RBC	NERBC	No Ag
in vivo: irr. TA3Ha	+	-	-	-	+	-
Epi:	+	ND	-	ND	ND	-

Summary of the Specificity Studies.

Ten million cells, generated in vivo or in vitro to the indicated antigen, were injected with the shown antigens in a local DTH foot pad assay in non-immunized mice. The DTH response significantly different ($P < .005$) is indicated as +, while - indicates a DTH reaction not significantly different from that elicited by primed cells alone. All experiments were done using 5 animals per group and repeated at least 3 different times with similar results.

ND = not determined.

Figure 1-9a:

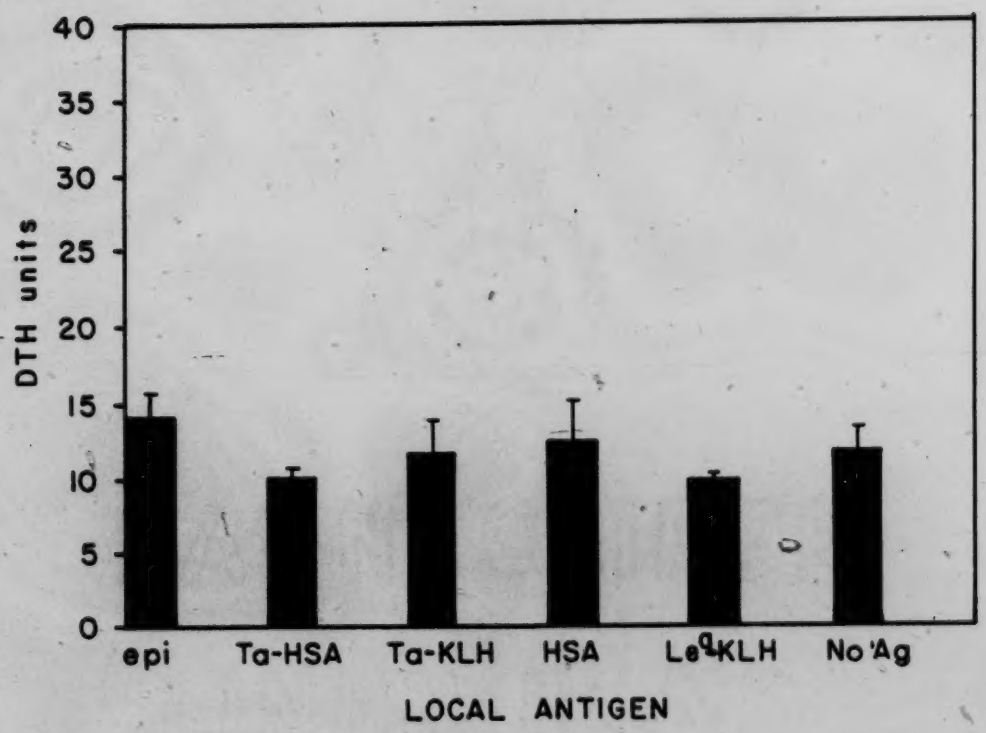


Contribution to the specificity by the carrier protein.

Epi primed cells 10^7 plus (a) epi, (b) TF(B) conjugated to HSA, (c) TF(B) conjugated to KLH, (d) HSA alone, (e) Le^a conjugated to KLH or (f) no antigen were injected in a local DTH assay. DTH was measured 24 hours later.

* $p < .005$ (Injection of primed cells alone was used as background for the comparison.)

Figure 1-9b:

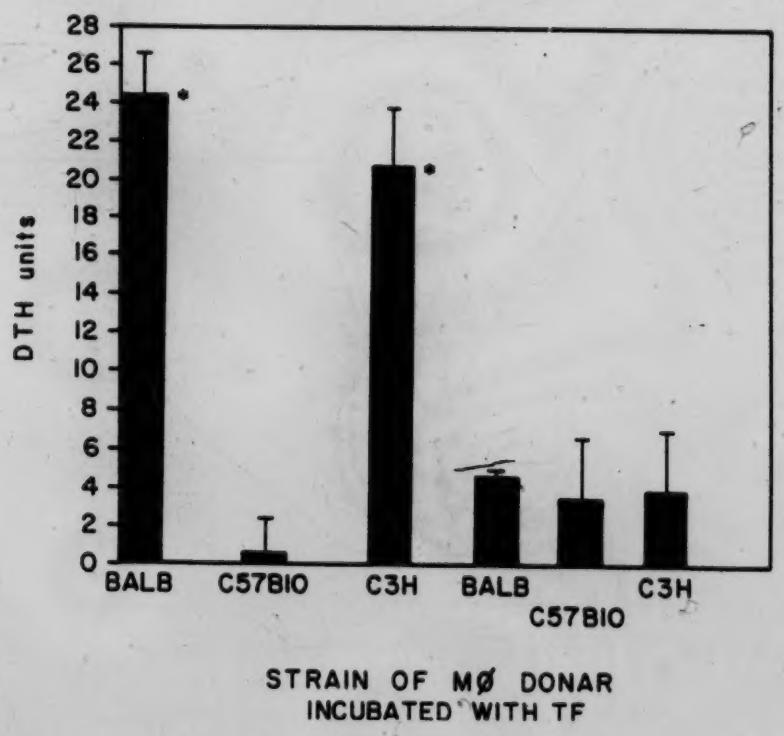


Contribution to the specificity by the carrier protein.

HSA primed cells 10^7 plus (a) epi, (b) TF(B) conjugated to HSA, (c) TF(B) conjugated to KLH, (d) HSA alone, (e) Le^a conjugated to KLH or (f) no antigen were injected in a local DTH assay. DTH was measured 24 hours later.

* $p < .005$ (Injection of primed cells alone was used as background for the comparison.)

Fig 1-10a:

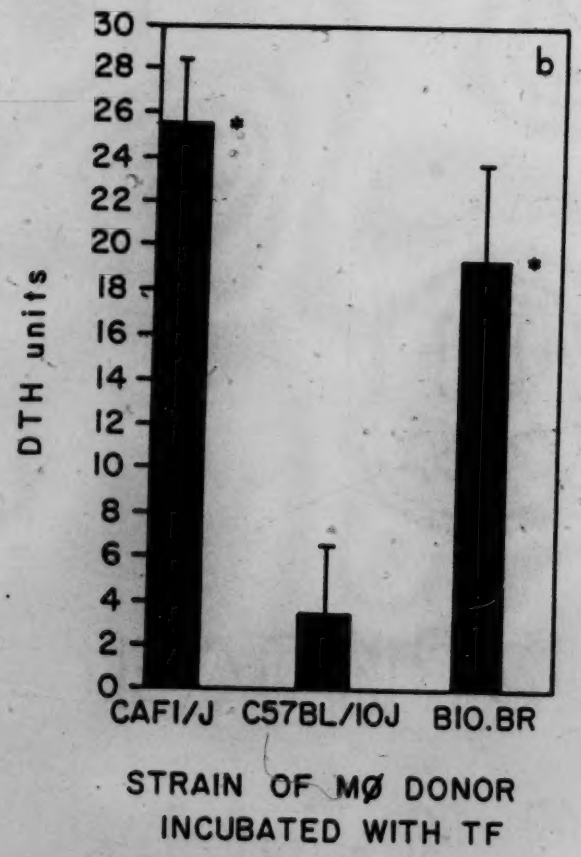


Restriction of the DTH response.

Ten million epi primed cells plus 10^6 TF(B)-HSA pulsed macrophages from the indicated strains were injected into the footpad of a non-immunized mouse. The DTH response was measured at 24 hours after the injection. The net DTH was calculated by subtracting from the DTH swelling in the experimental group (Primed cells plus macrophages pulsed with TF-HSA) the footpad swelling in the control group (Primed cells plus macrophages pulsed with HSA). Antigen (TF-HSA) pulsed macrophages were injected in the absence of primed cells to control for a possible difference in the background response to the macrophages of the different haplotypes.

* $p < .005$ (The swelling seen in the groups injected with antigen pulsed macrophages alone was used as background in the comparison.)

Figure 1-10b:

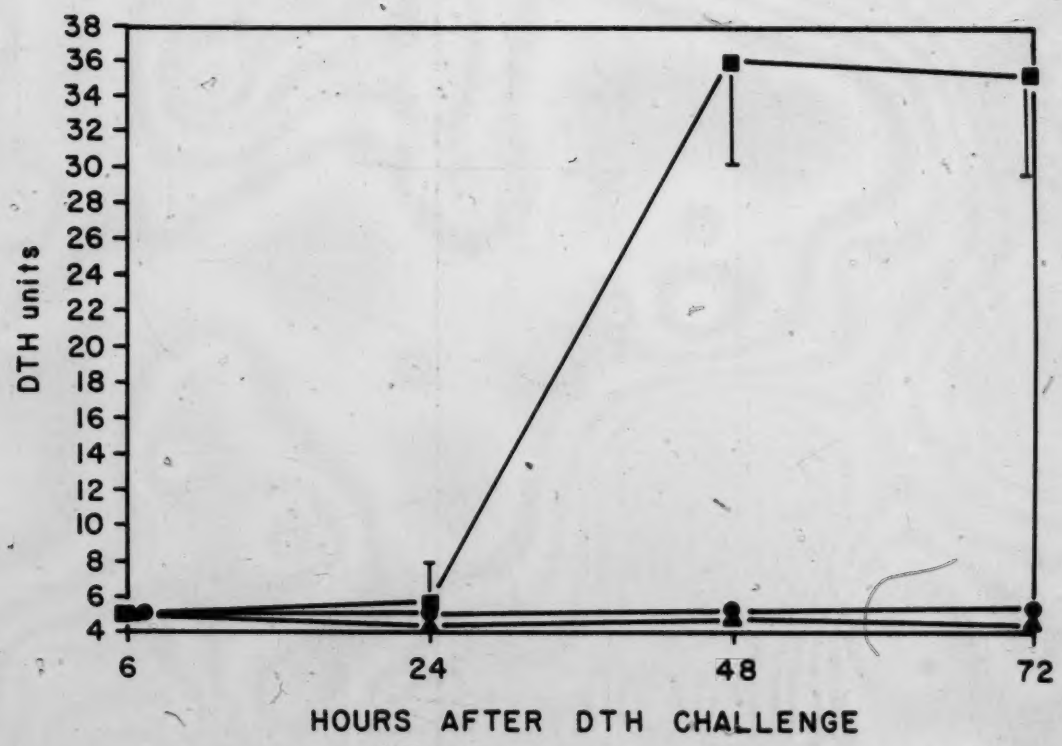


Restriction of the DTH response.

Ten million epi primed cells plus 10^6 TF(B)-HSA pulsed macrophages from the indicated strains were injected into the footpad of a non-immunized mouse. The DTH response was measured at 24 hours after the injection. The net DTH was calculated by subtracting from the DTH swelling in the experimental group (Primed cells plus macrophages pulsed with TF-HSA) the footpad swelling in the control group (Primed cells plus macrophages pulsed with HSA).

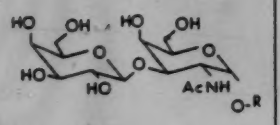
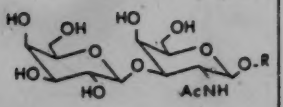
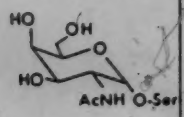
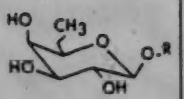
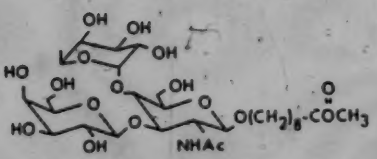
* $p < .005$

Figure 1-11:



Adoptive transfer of DTH effector cells. Normal unimmunized mice recieved 5×10^7 epi primed cells in an adoptive transfer followed by a foot pad challenge for DTH at (■) 4 hours and (●) 24 hours after the adoptive transfer. Controls (▲) were challenged for DTH without previous adoptive transfer of primed cells.

Table 1:

STRUCTURE OF TERMINAL IMMUNODOMINANT GROUPS	NAME	TRIVIAL NAME
	β-D-Gal-(1-3)-αGalNAc-O-$(CH_2)_8$-R	TF(α)
	β-D-Gal-(1-3)-βGalNAc-O-$(CH_2)_8$-R	TF(β)
	GalNAc-O-Ser-R	Tn
	6-deoxy-D-Gal-O-R	Fuc.
	β-Gal-(1-3)-β-GlcNAc-O-$(CH_2)_8$-$COCH_3$ (1,4 α-Fucose)	Lewis^a (Le^a)

The chemical structure of the synthetic antigens is shown along with the nomenclature. The Thomsen-Friedenreich (TF) antigen Gal-GalNAc- used in these studies were of both the TF alpha and TF beta conformation, referring to the linkage between the sugar ring and the carbohydrate spacer arm. Tn (a-GalNAc-Ser) is the immediate precursor to TF. Denoted as "R" is the carrier protein in this case either HSA or KLH.

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Chapter 3

Specific Anti-Tumor T-Cell Immunity Induced by a Tumor Associated Glycoprotein (TAG) and Defined Synthetic Tumor Associated Carbohydrate Antigens

INTRODUCTION

Mucins are high molecular weight glycoproteins with carbohydrate contents of 60-80% (1), normally secreted as biological lubricants by certain tissues (2). Monoclonal antibodies (MAbs) made against tumor tissue often recognize mucin associated carbohydrate determinants. For example mabs generated to tumor tissue detect antigens such as CA19-9, DuPan-2, CAI and CA 125 associated with tumor mucins (3,4,5,6,7). Tumor associated alteration of mucins may reflect changes in carbohydrate determinants resulting from 1) neoglycosylation as shown to occur with the 19.9 antigen, or 2) incomplete glycosylation revealing otherwise cryptic determinants such as the Thomsen-Freidenriech (B-Gal-(1-3)-GalNAc, TF) and the Tn (GalNAc-O-Ser) antigens.

That tumor associated mucin may shield the tumor from certain forms of immune destruction i.e. complement mediated antibody lysis and cytotoxic lymphocyte mediated killing (8,9) has been suggested. Examination of cultured human glioma cells and their ability to elicit an allogeneic MLR reaction suggest that a galactosaminoglycan (GAG) coat inhibits the interaction between the tumor cell and the lymphocyte. The observations suggest that the GAG coat may be responsible for the ability of the tumor to evade immune destruction (9). TA3Ha, a murine mammary adenocarcinoma cell line, produces a high molecular weight mucin-like glycoprotein , epiglycanin (epi) (10). Epi is a 500,000 mw glycoprotein, which is approximately 80% carbohydrate and carries multiple

immunodominant TF and Tn determinants (10). A number of reports suggest that epi may inhibit the immune detection of H-2 determinants on the TA3Ha cells and thereby allow the cells to evade destruction. This phenomenon is reflected in the ability of TA3Ha cells to grow in an allogeneic host, while the cell line from which TA3Ha was derived lacks both epi and the ability to grow in an allogeneic host (8,11)..

We have provided evidence to indicate that T (DTH) cells can recognize carbohydrate determinants. We demonstrated that T (DTH) cells primed with epi could recognize and respond to synthetic TF and Tn carbohydrate haptens linked to an appropriate carrier (hereafter refered to as a synthetic tumor-associated glycoconjugate, s-TAG) but not to the carrier alone nor to irrelevant carbohydrate haptens linked to the carrier. Similarly cells primed with TF or Tn s-TAG could recognize and be triggered by epi.

Based on the characteristic amplification of the final and non-specific effector mechanism in a local DTH reaction, we reasoned that DTH reactions initiated by T-cells recognizing determinants on mucin molecules may provide an effective immune defence mechanism against a mucin covered tumor. Given that mucins are highly glycosylated proteins and that tumor associated mucins have altered carbohydrate structures, a DTH reaction specifically directed to the carbohydrate determinant on the mucin may represent a particularly effective anti-tumor response.

In this paper we provide evidence that a cell population primed to epi significantly delays tumor growth. The data presented shows a significant delay in tumor growth associated with a cell population primed

with epi or with s-TAGs carrying TF or Tn epitopes, but not with cell populations primed with the carrier or the carrier conjugated to an irrelevant carbohydrate hapten.

METHODS:

GENERATION OF AN ANTI-TUMOR EFFECTOR CELL POPULATION.

An anti-tumor effector cell population was generated by the culture method previously described for the generation of DTH effector cells, see chapter 2.

ANITBODY TREATMENT of EFFECTOR CELLS

A primed cell population was obtained as described above ,washed, counted and divided into separate tubes for the antibody treatment. The cells were treated with (1) anti Thy1.2 antiboby (at 1/1000 diln., obtained from NEN), (2) anti Lyt1.2 antibody (at 1/1000 diln.,obtained from NEN) or (3) anti Lyt2.2 antibody (at 1/1000 diln., obtained from NEN.) as described previously, see chapter 2.

SYSTEMIC DTH ASSAY

Cells harvested from six day cultures were washed with RPMI and counted. The primed cells were adoptively transfered to unimmunized mice at 5×10^7 cells/mouse. The animals were challenged for DTH by a local foot pad injection of irradiated TA3Ha cells (10^6 cells in 25 ul PBS). Controls were challenged for DTH without previous adoptive transfer. At 24 and 48 hr after the injection , the thickness of the right and the left

hind foot pads were measured with an oditest (0-10mm,0.01mm) thickness guage (obtained from H.C Kroplin, W Germany) and the DTH swelling estimated by subtracting the thickness of the uninjected foot pad from that of the injected one.

DTH unit = 10^{-2} mm

WINN ASSAY

DTH primed cells were induced as described above. Ten million primed lymphocytes from the spleen cell cultures were mixed with 1-2 x 10^5 live tumor cells and injected s.c. into the foot pad. The control mice were injected with HSA primed or normal spleen cells and tumor cells. Foot pad swelling was measured at 12, 24, 48 and 72 hours and every 2 days there after. To estimate the time at which any swelling caused by a DTH reaction would be minimal, mice were injected with a mixture of primed or normal cells with irradiated tumor cells. Any swelling seen in this group was attributed to be causes other than tumor growth for example a DTH reaction and when present subsided by 96 hours after the injection. Tumor growth was measured every 2 days starting 48 hours after the peak DTH swelling. DTH and tumor size were measured an oditest thickness gauge and the increase in foot pad thickness estimated. Tumor size is indicated as the difference in thickness between the injected and the uninjected foot pad.

Units = 10^{-2}mm

SYSTEMIC ADOPTIVE TRANSFER OF EFFECTOR CELLS

Fifty million primed cells were transfered to normal recipients via an iv injection (in .5-1ml PBS). A local foot pad challenge with tumor cells was done immediately, and tumor growth was monitored as described for the local Winn assay.

IN VIVO IMMUNIZATION.

CAF_1/J mice were immunized with indicated doses of epi or synthetic antigens in 50% CFA ip. Ten days later these animals were challenged for DTH by a sc foot pad injection of 10^6 irradiated TA3Ha cells in 25 ul PBS. DTH was measured at 24-48 hours after the challenge, and serum samples were collected at that time.
The animals were then challenged with 10^3 live TA3Ha cells ip. and survival monitored.

EXTRACTION OF EPIGLYCANIN

Epiglycanin (epi) was extracted from the ascites fluid of TA3Ha tumor bearing mice as previously discribed, see chapter 2.

EXCISION PRIMING FOR PROTECTION

Mice were injected [illegible] TA3Ha cells at a local subcutaneous site in the lower neck re[illegible] the tumor was palpable, it was removed

by surgical excision under general anesthetic (Mehtophane). After excision, mice were left for 1-2 weeks to recover, then challenged with a live tumor and survival was monitored.

HISTOLOGY

Frozen sections of the local inflammatory site or the site of tumor growth were prepared using standard histology techniques. Tissue samples of local inflammatory sites were taken and frozen immediately in liquid nitrogen. The samples were cut using a "Tissue-Tek" Cryostat, transfered to Methanol/Ether (1:1) and stained with Hematoxylin and Eosin.

TUMOR CELL LINES

The TA3Ha cell line (15) was kindly provided by Dr.J F Codington (Mass. General Hospital, Boston MA.) and passaged weekly as an ascites in (Balb x A/J) F1 mice. Each ascites line was terminated after in vivo passage 10 and a fresh frozen aliquot used to start a new ascites line.

The mKSA tumor cell (16) line was provided by Dr. P.A. Bretscher and carried similarly to the TA3Ha cell line.

RESULTS

Inhibition of tumor growth by cells primed in vitro with epi.

A local Winn assay was used to test whether cells generated in the in vitro system, under conditions for optimal generation of DTH effectors, were able to inhibit tumor growth. Cells primed to epi were mixed with live TA3Ha cells and injected into the footpads of unimmunized mice. A significant delay in tumor growth was observed in the experimental group as compared to various controls including those which received an injection of cells primed with HSA, or normal spleen cells mixed with live TA3Ha cells.

A systemic adoptive transfer assay was used to determine if the in vitro primed cells were able to home to the tumor. Fifty million primed cells were transfered iv into normal recipients and the foot pad injected with live TA3Ha cells. Figure 2-2 , shows a marked reduction in tumor growth in the adoptively transfered animals as compared to animals which did not receive an injection of primed cells but were injected in the footpad with live tumor.

Tumor specific inhibition.

The possibility that the epi or sTAG primed cells inhibited tumor growth in a non-specific manner was ruled out by the following

experiment. A local Winn assay was performed using an anti epi primed cell population and a tumor cell line which does not carry epi cross reactive antigens, the SV40 transformed fibrosarcoma line , mKSA. Figure 2-3 shows that the growth of mKSA tumor was not significantly altered by the anti-epi primed cell population, while TA3Ha tumor growth was delayed by the same cell population.

Cell surface phenotype of anti-tumor effector cells primed in vitro with sTAG

The Winn assay was used to determine the cell surface phenotype of effector cell populations induced by defined synthetic carbohydrate antigens (s-TAGs). Effector cells primed with synthetic TF(a) antigen were mixed with live TA3Ha cells and injected into the foot pad of unimmunized mice. Characterization of the cell surface phenotype of the effector cell was done by treating the TF(a) primed cell population with either monoclonal anti-Thy1, anti-Lyt1 or anti-Lyt2 antibodies in the presence of complement immediately prior to the Winn assay. Figure 2-4 demonstrates that the inhibition of local tumor growth was abrogated by anti-Thy1 and anti-Lyt1 treatment of the effector cell population in the presence of complement, but not by complement treatment alone nor by treatment with anti-Lyt2 antibody and complement. This suggests the existence of a T dependent anti-tumor response.

Histology of the Winn assay site.

To confirm that the increase in foot pad measurement seen at one week

after injection was due to tumor growth frozen sections of the local Winn assay site were made. Normal mice were injected with a mixture of primed cells and tumor cells, or primed anti-Thy1 + C' treated cells and tumor cells. Figure 2-5 shows the presence of a large number of tumor cells in site injected with the anti-Thy1 treated cell, suggesting the Thy1+ cells are central to the inhibition of the tumor growth.

Systemic immunization for protection.

To further test the potential of epi and the s-TAG antigens to protect against tumor growth, animals were immunized with various amounts of antigen emulsified in 50% CFA (ip). Ten days later, they were challenged with a live TA3Ha tumor ip and survival was monitored (table 1). Mice immunized with relatively low doses of either epi or TF(B)-HSA showed an increased resistance to tumor growth while immunization with Glu-BSA or HSA had no effect on the resistance to tumor growth. Occasionally animals which had survived the tumor challenge were given a second tumor challenge, similar to the first 3-4 months later. This challenge was also resisted (data not shown).

DISCUSSION

Based on the characteristic amplification during the DTH reaction and the non-specific effector function, we propose that this class of immune response may be a valuable anti-tumor immune mechanism. A DTH reaction directed at a mucin determinant may be particularly effective in bringing about inhibition of tumor growth, particularly where mucins appear to shield the tumor from lysis by immune responses directed at conventional cell surface antigens (8,9,11).

To test this hypothesis we utilized the TA3Ha murine mammary adenocarcinoma, as a tumor model. TA3Ha cells synthesize a mucin-like 500,000 m.w. glycoprotein , epiglycanin (epi), as a major component of its cell surface glycocalix. Epi is approximatly 80% carbohydrate in composition and carries the immunodominant TF (B-Gal-(1-3)-a-GalNAc) and Tn (a-GalNAc-O-Ser) structures (10). It has been reported that the presence of epi effectively shields the tumor cells from destruction by immune responses directed against H-2 determinants, allowing the TA3Ha cell line to grow in an H-2 incompatible host (8,11). We attempted to confirm this claim by testing TA3Ha cells for susceptibility to lysis by cytotoxic lymphocytes directed against its H-2 determinants . These results (not shown) suggested that TA3Ha cells are both resistant to lysis by cytotoxic lymphocytes primed to H-2 antigens and are unable to induce an anti H-2 CTL population.

To determine the anti-tumor effect of cells primed in vitro to epi, the primed cells were injected together with tumor cells in a local Winn

assay. These cells were able to delay the growth of the TA3Ha tumor cells. The inhibition was not complete, and the tumor injected together with the primed cells eventually grew to the same size as the control tumors, that is tumor cells injected with unprimed spleen cells or together with spleen cells primed to an irrelevant antigen. The histology of the tumor site show a reduction in the number of tumor cells when the inoculum included primed T cells, figure 2- 5. These findings suggest that the primed T cells inhibit some tumor growth but cannot totally eradicate the tumor.

An adoptive transfer assay was used to test the ability of the in vitro primed cells to inhibit tumor growth. Epi primed cells were transfered i.v. and followed by an immediate local tumor challenge. We observed a delay in tumor growth in such adoptively immunized mice, relative to controls which received tumor cells only. These results suggest that the in vitro DTH primed cells are able to home to a tumor or alternatively that DTH primed cells induced a secondary effector function which inhibits the tumor. Further experiments are needed to discriminate between these two possibilities.

That DTH mediated tumor inhibition was specific for the tumor antigen was shown in experiments in which the priming antigen was HSA. Cells cultured in the presence of HSA did not inhibit TA3Ha tumor growth in a local Winn assay, suggesting that DTH induced tumor regression is dependent an antigen specific immune response. Alternatively one may speculate that epi primed cells inhibit tumor growth non-specifically. This was tested by attempting to inhibit the growth of a tumor which does not express epi cross reactive antigenic determinants with the epi primed cells. The SV40 transformed fibroblast cell line, mKSA, was used. Excision

priming experiments (personal communication, P.A.Bretscher) showed no cross priming between TA3Ha and mKSA cells. No significiant inhibition or delay of the mKSA tumor growth by the epi primed cells was observed while the TA3Ha tumor growth was inhibited. Thus the effector cell population involved in the inhibition of TA3Ha tumor growth appears to be antigen specific.

To determine if DTH directed against a mucin associated carbohydrate determinant, the effector cells were primed in vitro with the s-TAG TF(B)-HSA. Cells primed with TF(B)-HSA were divided and treated with anti-Thy1, anti-Lyt1 or anti-Lyt2 monoclonal antibodies in the presence of complement then used in the local Winn assay together with TA3Ha cells. A Thy1+ Lyt1+ Lyt2- cell population was capable of mediating a significant delay in the tumor growth. The cell surface phenotype of the effector cell population was found to be identical to that of the DTH mediating cell which may be generated under the same priming conditions. Histological sections of the local Winn site also suggest the inhibition of tumor growth is dependent on a Thy1+ cell. The data presented here support the hypothesis that a DTH reaction directed against a mucin associated carbohydrate determinant may provide an effective anti-tumor immune mechanism.

The inhibition of tumor growth seen in the adoptive transfer experiment was temporary, that is, the tumors in the experimental groups eventually recovered growth to the same size as the controls. Considering the extreme lethality of the TA3Ha cell line it is possible that this was due to the relatively large tumor burden used. The extreme lethality of

TA3Ha cell line has been shown by Lippman (17) where the average survival time after ip inoculation of 10^1 TA3Ha cells was 17 days.

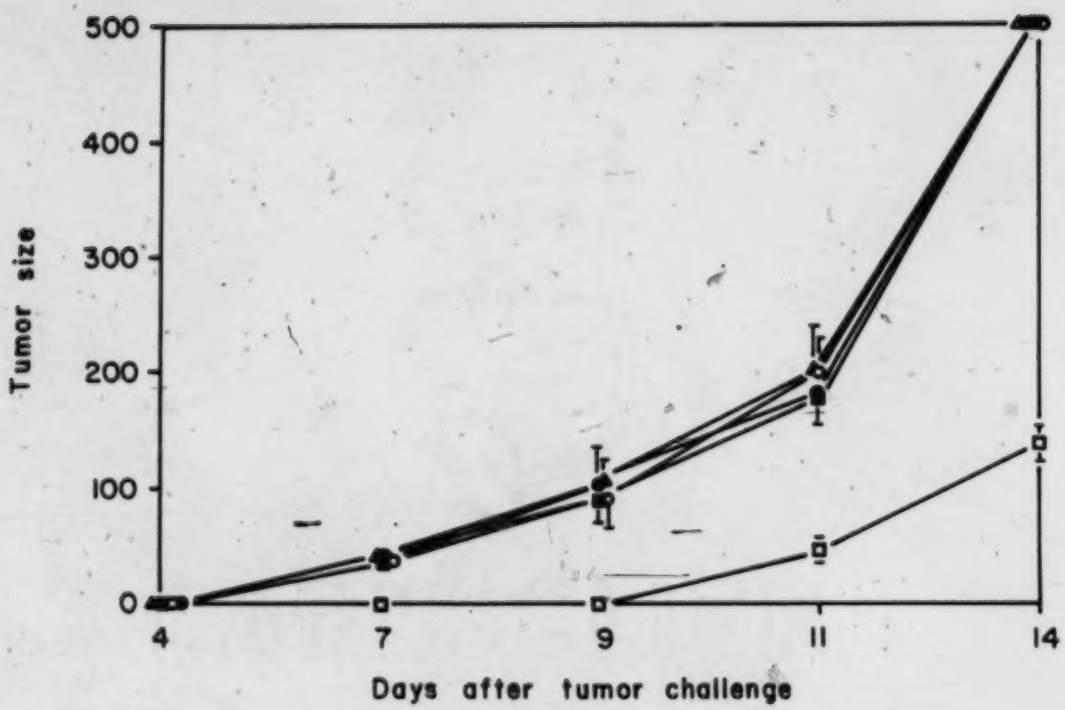
Alternatively we propose that the primed cells may be suppressed by the host or possibly cleared from circulation a short time after transfer. The cell population which mediates the delay of tumor growth correlates with the DTH mediating cell population both in cell surface phenotype and in induction requirements. We have previously reported that following iv transfer of epi primed cells DTH activity can be demonstrated by an immediate local DTH challenge but not if the challenge is delayed by 24 hours. This observation suggests that primed cells may be somehow inactivated following iv. injection. The mechanism of this inactivation was not addressed but may involve active suppression of the effector cells or may be by means of cell clearance.

The potential of s-TAGs to immunize against the tumor was tested through a direct, in vivo immunization followed by a live tumor challenge. Long term effective immunity was induced by direct in vivo pre-immunization. The protection appears to be long term, and some animals which were given a second tumor (10^3 TA3Ha cell, ip.) challenge 4 months after the first were able to reject the subsequently induced tumors (data not shown). This is in contrast to the adoptive transfer of primed cells, where tumor inhibition appeared to be temporary. However, no direct correlation between tumor protection and DTH was established in the in vivo immunization experiments. Whatever the mechanism, it is specific and dependends on the immunizing antigen.

Many cancers of epithelial origins such as ovarian, colon and breast

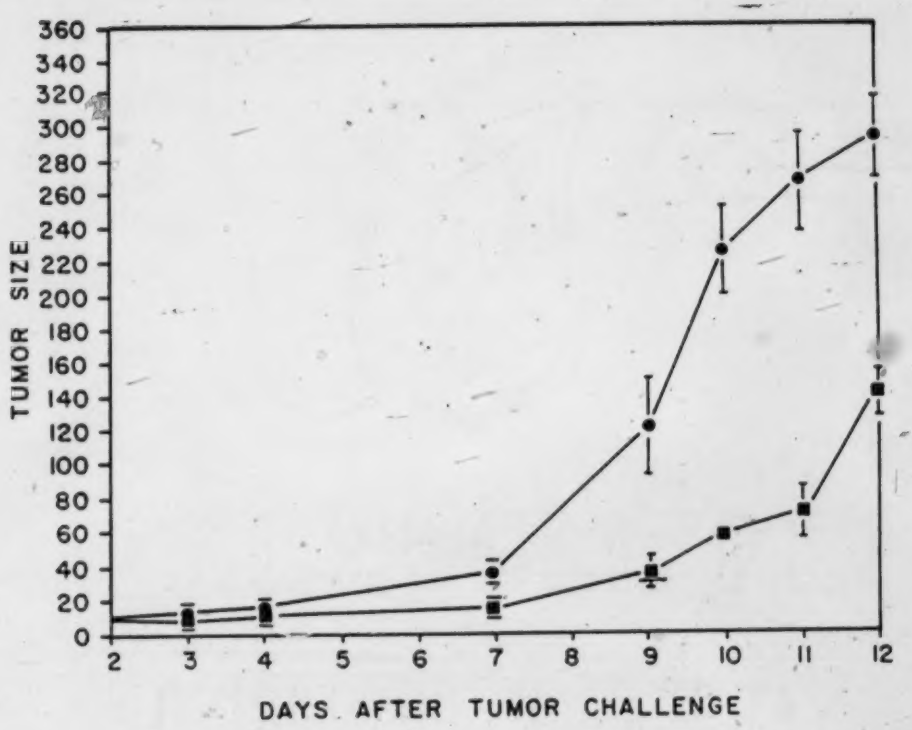
cancers produce high molecular weight glycoproteins called mucins (12). Mucins are present in normal seroviscous secretions, and can function to provide a barrier to protect cells against physical and osmotic damage. Tumor associated mucins may have a similar protective role, as is suggested by animal models (8) and by clinical observations of human tumors (9). Mucins may inhibit immune recognition of tumor cell surface antigens and thus the destruction of tumor cells by mechanisms such as complement-antibody mediated lysis and cytotoxic lymphocyte mediated killing. On the other hand, many tumor associated antigens, such as CA19-9, DuPan-2, CAI and CA125 (3,4,5,6,7), are carbohydrate determinants on mucins. We reasoned that an immune response with an amplified and non specific anti-tumor effector component such as a DTH response, directed at these mucin associated carbohydrate determinants may be able to inhibit tumor growth. The experiments reported here are encouraging in that they provide the first demonstration of a DTH cell population , induced by a tumor associated mucin like molecule or its synthetic carbohydrate epitopes, capable of mediating a tumor inhibitory function. The final effector mechanism inhibiting tumor growth was not addressed in these experiments. The cell population which mediates the TAG specific DTH correlates nevertheless with the cell population which inhibits tumor growth in an adoptive transfer assay. The cell populations were induced under the same priming conditions and both activities were mediated by a Thy1+ lyt1+ lyt2- cell population. We feel that further research into the use of s-TAGs as immunogens may lead to new approaches in the management of human malignant diseases.

Figure 2-1:



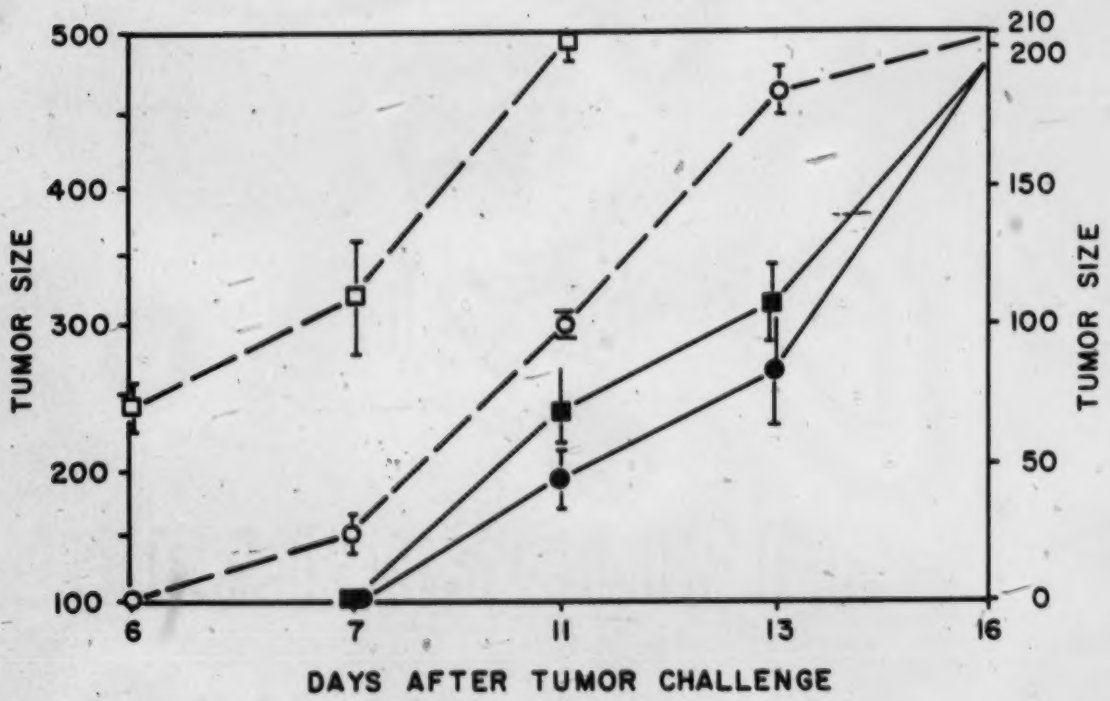
Inhibition of tumor growth by epi primed cells. Ten million cells primed with epi in vivo and in vitro (□) were able to significantlly delay the growth of TA3Ha tumor cells in a local Winn assay as compared to cells primed with epi in vivo only (○), HSA in vivo and in vitro (△), non primed cells cultured with epi (■) or normal spleen cells(●).
Tumor size was measured in units of 10^{-2}mm.

Figure 2-2:



Adoptive transfer of epi primed cells. Epi primed cells (5×10^7) were adoptively transfered (■) into non-immunized syngeneic mice. Immediately following the transfer these mice were challenged with live TA3Ha cells at a subcutaneous site. Control (●) mice received a tumor challenge only. The tumor growth was significantly delayed in the adoptively transfered animals. Tumor size was measured in units of 10^{-2}mm.

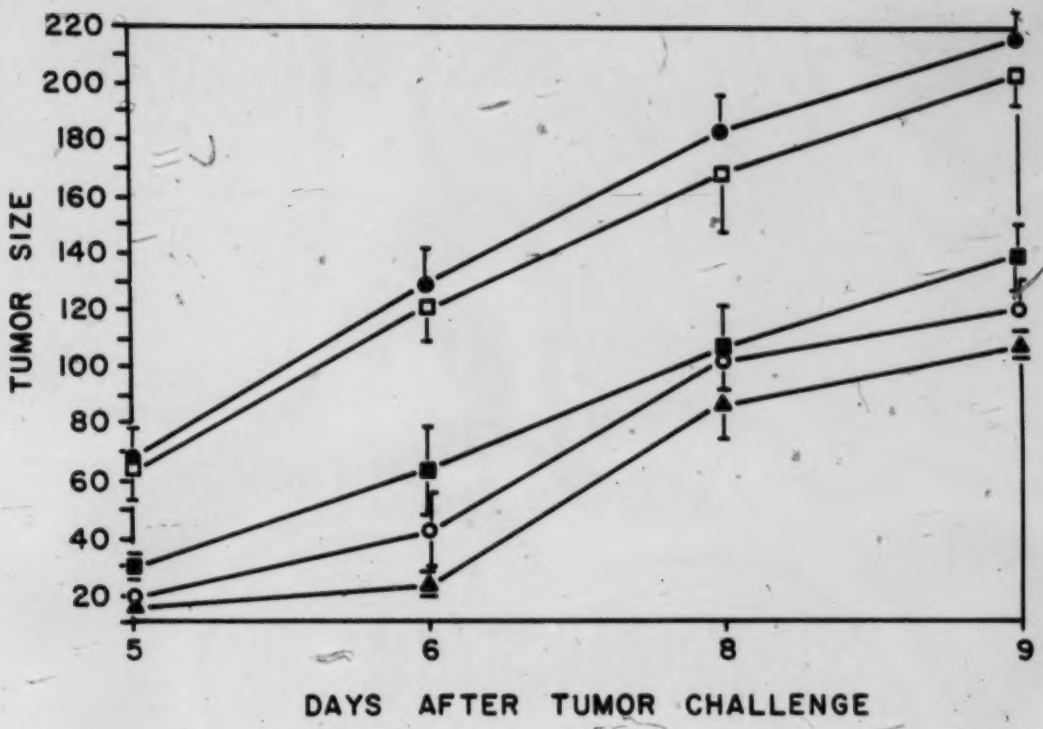
Figure 2-3.



Specificity of tumor inhibition. Epi primed cells (5×10^7) were adoptively transfered to normal syngeneic mice, which were subsequently given a subcutaneous challenge of (○) 2×10^5 live TA3Ha cells or (●) 2×10^5 live mKSA cells. Controls received the tumor challenge (□) TA3Ha and (■) mKSA only. The size of the tumors were measured at various times after challenge. Tumor size was measured in units of 10^{-2} mm.

* (□) differs from (○) $P < .001$

Figure 2-4:



Cell surface phenotype of the anti-tumor effector cell primed in vitro with s-TAG.

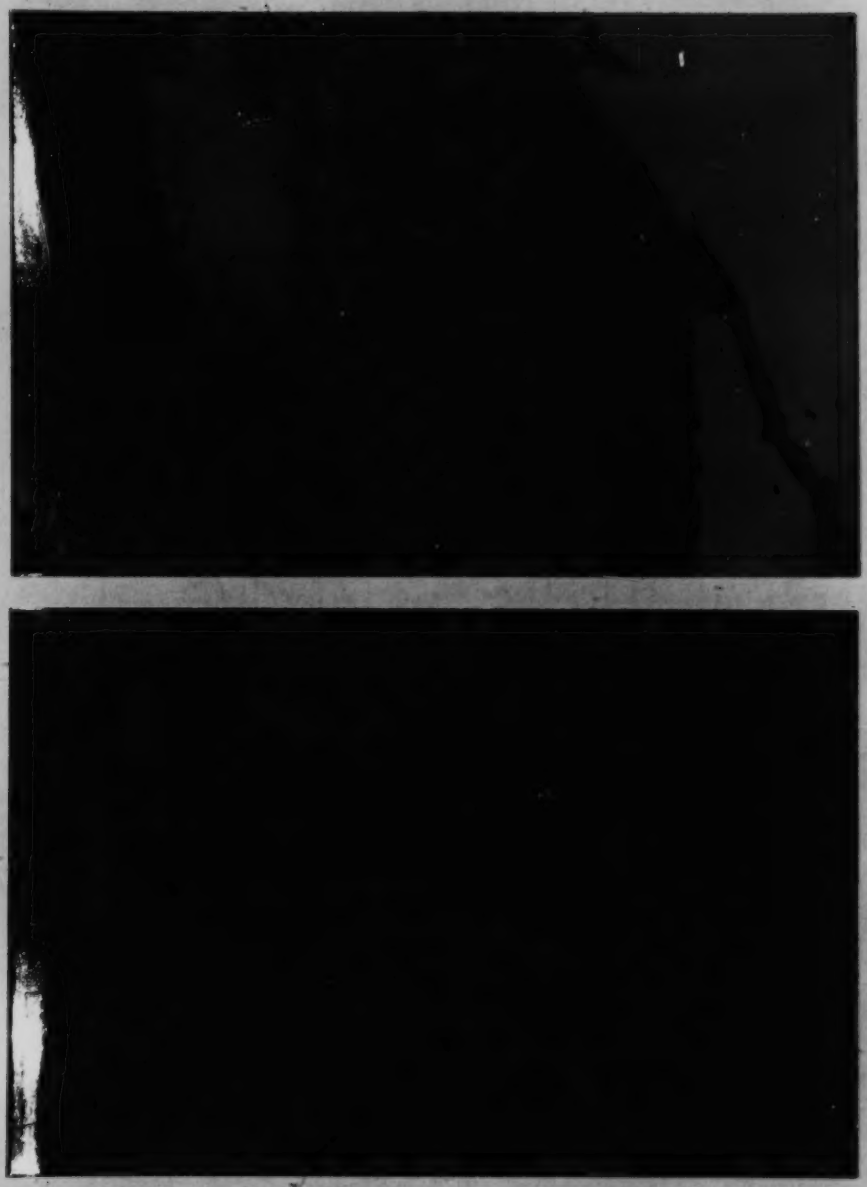
Cells primed with TF were treated with (●) anti-Thy1, (□) anti-Lyt1 or (■) anti-Lyt2 antibody in the presence of complement or (○) with complement alone. Group (▲) was not treated at all. The treated cells were mixed with 2 x 10^5 live tumor cells and the mixture injected into the footpad of normal mice. The tumor growth was measured at various times thereafter. Tumor size was measured in units of 10^{-2} mm.

(●)and (□) differ from (■),(○) and (▲). $P < .001$

Figure 2-5a :

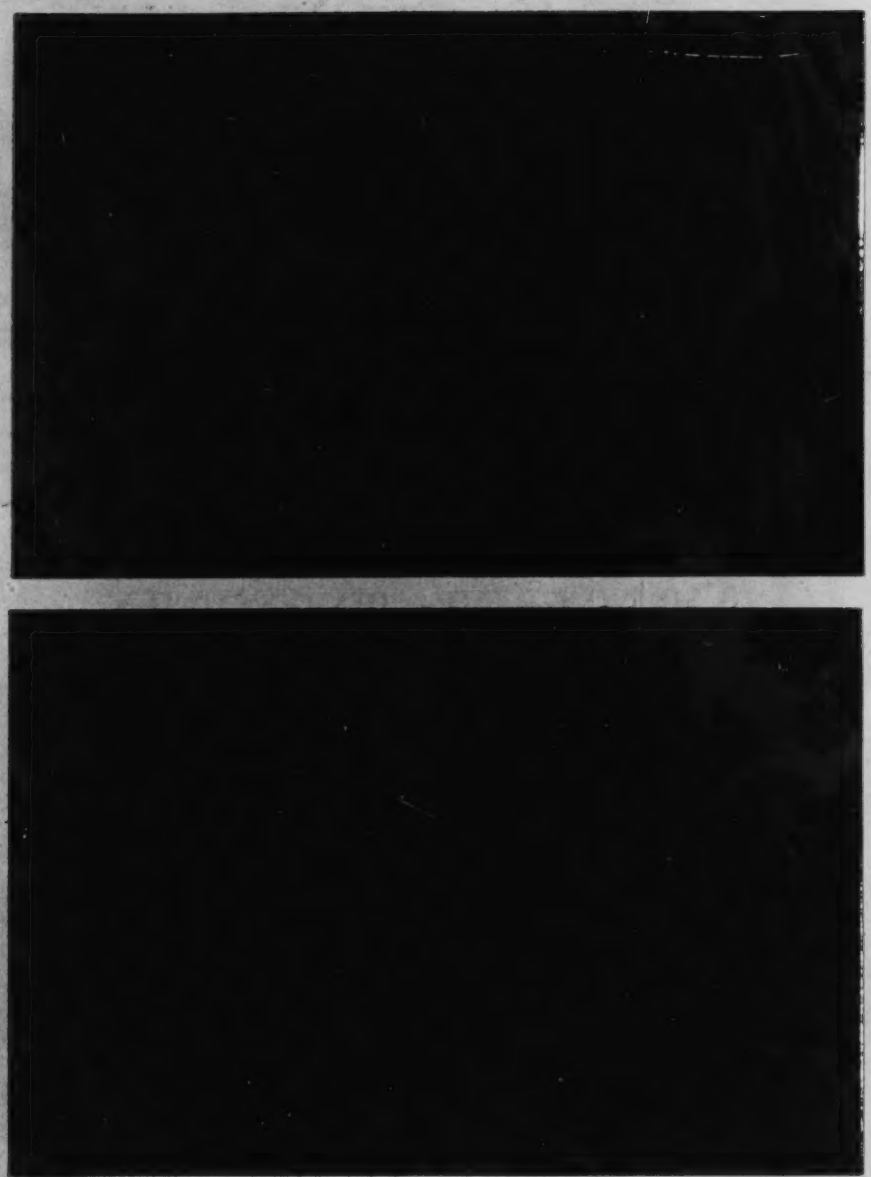
Histology of the local Winn assay site. 5-7 days after injecting primed nontreated cells plus live TA3Ha cells the local tissue was frozen, sectioned and stained by standard techniques with Hematoxylin-Eosin stains.

Figure 2-5b:



Histology of the local Winn assay site. 5-7 days after injecting primed anti-Thy1 and C' treated cells plus live TA3Ha cells the local tissue was frozen, sectioned and stained by standard techniques with Hematoxylin-Eosin-stains.

Figure 2-5c:



Histology of the local Winn assay site. 5-7 days after injecting Ta3Ha tumor cells the local tissue was frozen, sectioned and stained by standard techniques with Hematoxylin-Eosin stains.

Table 2-1:

Dose:	Immunizing antigen: (# alive/total at 4 weeks.)		
	EPI	TF-HSA	Glu-BSA
0.1ug	1/20	4/15	0/10
0.5ug	5/20*	7/15**	0/10
1.0ug	8/20**	5/15*	0/10
5.0ug	8/15**	6/15**	0/10
10ug	7/20**	3/15	0/10
cont.	0/20	0/15	0/10

Direct immunization for systemic protection. Mice (Balb xA/J) F1 were immunized with varying doses of epi, sTAG or Glu-BSA emulsified in 50% CFA and injected i.p.. 10 days later the immunized animals were challenged with 3 x 10^3 TA3Ha cells i.p. and survival was monitored.

* $P < .025$

** $P < .01$

*** $P < .005$

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Chapter 4

Discussion

My aim was to determine (1) the ability of T cells to recognize carbohydrates as antigenic determinants and (2) the affect on tumor growth of a T cell reaction, specifically DTH, directed to a carbohydrate antigen on a tumor associated mucin. These questions were addressed using a purified tumor associated mucin like molecule, epi, and synthetic epitopes of the carbohydrate determinants present on epi.

T cell responses directed against carbohydrate determinants

Characterization of antibody recognition of carbohydrate antigen is well established (1). However, T cell recognition of carbohydrate determinants has been difficult to resolve. Results from studies attempting to resolve the controversy have been open to variable interpretations. The difficulty encountered in the T cell system may derive from the complex interactions involved in T cell antigen recognition. The requirement for processing and presentation of antigen in order for a productive T cell response to occur complicates the interpretation of the data. Frequently used experimental approaches involving alteration of undefined cell surface carbohydrates may in fact be establishing the requirements for the complex molecular interaction

leading to a T cell response, rather than the ability of the T cell to recognize carbohydrate antigens.

In contrast to earlier studies of T cell responses to carbohydrate antigens this study was focused on the T cell recognition specificity carbohydrate determinants as nominal antigen. This was accomplished using synthetic carbohydrate epitopes coupled to various protein carriers. Synthetically constructed antigens (CHO-Protein carrier) are idealy suited to determine the T cell antigen recognition specificity assuming that the carrier proteins, HSA and KLH, are processed by the antigen presenting cell in a similar manner. The carbohydrate epitope and protein carrier can be varied and the contribution of each to the T cell antigen recognition specificity can be determined.

The DTH reaction as used in these studies represents a very sensitive assay for cell mediated immunity to carbohydrate antigens. Marchel (1) demonstrated the extreme sensitivity of the DTH assay using limiting dilutions of primed cells and T cell clones in a local foot pad assay. The experiment suggested that one primed T (DTH) effector cell can elicit a detectable DTH reaction. The local DTH assay may therefore be more sensitive that the more commonly used proliferation and IL-2 release assays. Recent evidence also suggests that T cell <u>in vitro</u> antigen specific proliferation may not be a direct correlate of the <u>in vivo</u> effector function and antigen specificity (D. Lynch personal communication).

My initial efforts concentrated on the generation and detection of a carbohydrate specific DTH response. In early <u>in vivo</u> experiments immunization with TA3Ha tumor cells induced a DTH response that could be

elicited either by irradiated TA3Ha or NERBC, but not by normal red blood cells nor by an irrelevant tumor, L1210. The neuraminidase treatment of human red blood cells removes the terminal NANA on the glycophorin molecule and thus reveals TF determinants. The TF antigen is thus exposed on the NERBC but not on the normal RBC, nor on the L1210 cells (3). It was therefore likely that this response was directed towards the TF carbohydrate determinant.

In Vitro generation of DTH

An in vitro priming method for induction of carbohydrate specific DTH effector cells was developed, based on Bretscher's demonstration of the role of cell density and antigen concentration during in vitro immune class regulation (4). In vivo primed spleen cells cultured at low cell density with a relatively low concentration of antigen provided an efficient way of obtaining specifically primed cells. Priming in vitro was antigen dependent and shown to result in activation of a Thy 1+ Lyt 1+ Lyt 2- cell population. The kinetics of the local inflamatory response indicated a maximal swelling reaction within 24-48 hours of injection and was deemed characteristic of DTH, rather than an antibody mediated Arthus type response. Histological sections of the site showed a massive infiltrate of monocytes and neutrophils as described for murine DTH reactions (5). Thus the in vitro priming with epi or sTAGs induced a cell population which was shown to mediate a local DTH reaction.

Antigen speicicity of the DTH response.

Epi primed cell populations were tested in a foot pad assay for antigen recognition specificity by their capacity to elicit DTH. The local DTH reaction was elicited by epi, synthetic TF (both alpha and beta configuration) and Tn- conjugated to HSA and by TF conjugated to KLH but not by the inappropriate carbohydrates linked to the carriers, such as fucose conjugated to HSA or Le^a conjugated to KLH. Similarly TF and Tn primed cells were elicited by epi, TF and Tn conjugated HSA. Again neither fucose-HSA nor HSA alone elicited the local reaction. Fucose (6-deoxy-Gal) differs from the other synthetic antigens in that 1) it lacks the OH group in the 6th position and 2) it is linked to HSA without the NAc group present in the GalNAc. Thus it seems the OH and/or the NAc groups may be involved in creating the antigenic determinant recognized by the T (DTH) cells. Studies on the structural requirement of carbohydrate recognition by monoclonal antibodies and lectins suggest that hydrophobic interactions between the protein and the carbohydrate are important (6,7). The creation of a hydrophobic interaction may involve intramolecular hydrogen bonding in the carbohydrate molecule, such that certain hydroxyl and NAc groups may be critically important in the formation of the appropriate CHO conformation.

The possiblity that the specificity of the response also involves the protein portion of the molecule was further explored. It appears that HSA, the protein carrier to which the TF and Tn synthetic haptens are linked, does not contribute much to the specificity of the response. The cell population primed to epi, devoid of any HSA determinants as tested by

antibody, can be elicited by synthetic TF or Tn conjugated to HSA. Similarly a cell population primed to the synthetic hapten conjugated to HSA can be elicited by epi. Furthermore the Tn-HSA and the TF-HSA synthetic antigens differ in their linkage to HSA (see table 1-1). Tn is O-linked to a serine residue which is then coupled to HSA, while TF is linked to HSA via an eight carbon chain $(CH_2)_8$. Since DTH recognition does not discriminate between Tn, TF and epi it seems unlikely that the linker arm or the carrier provides a major contribution to the recognition specificity. This argument is supported by a second set of experiments where both TF-HSA and TF-KLH are able to elicit the epi primed response.

It is possible that the antigen requirements for the generation of DTH effector cells differ from that of the T cell antigen recognition requirement as determined by the local DTH reaction. The protein portion of the molecule, although not involved in determinig the antigen specificity , may be critical to the induction of the DTH response. Possibly a protein carrier is required in order for the antigen to be presented by the antigen presenting cell, while the DTH T cell may recognize the carbohydrate portion of the molecule. In light of recent studies by Berzofsky (6) it appears that a hydrophobic portion is necessary in order for an antigen to be properly presented, possibly in order for the antigen to associate with Ia on the cell surface. Carbohydrates are generally hydrophilic molecules, and although some hydrophobic interactions can occur (7), they may be insufficient to form the neccessary hydrophobic interactions required for presentation by antigen presenting cells as free carbohydrates. This intepretation is consistent with the claims stating that "in experiments where pure carbohydrate was used no DTH was observed" (8).

MHC restriction.

T cell responses, including DTH responses, to proteins and undefined cellular antigens have been shown to to be restricted to recognize antigens in the context of self encoded molecules (9). To address the question of restriction of T cell responses to carbohydrate antigens macrophages of different genetic origins were pulsed with TF-HSA and then injected in a local DTH assay together with epi primed cells. Only TF-HSA pulsed macrophages which shared identity with the T-cell population at the H-2 loci were able to elicit the DTH reaction. The data suggest that, similar to T cell responses to proteins, the T (DTH) response to carbohydrate antigens is MHC restricted. The observed MHC restriction of the carbohydrate specific responses may in fact be determined by the carrier protein.

The experiments reported here provide the first conclusive evidence that a T cell population can recognize a carbohydrate epitope. The role of the protein carrier in determining the specificity of the DTH response appears to be minimal, but a role of the protein carrier for antigen presentation is possible.

Relevance (to tumor growth) of the T cell response to tumor associated mucins

Many cancers of epithelial origin secrete mucins. Tumor associated mucins often differ from their normal counterparts in carbohydrate

structure. Recent studies suggest differences in carbohydrate structure and distribution between normal, preneoplastic and neoplastic colon associated mucins (10). Under normal conditions, mucins may function as protective barriers against physical damage. Studies using the murine TA3Ha tumor model suggest that epi, a mucin-like molecule, shields the TA3Ha cells from destruction by immune responses directed against conventional transplanation antigens, such as H-2 antigens. This is seen by the fact that TA3Ha cells can grow in an H-2 incompatible host (11) and that they cannot be lysed by anti $H\text{-}2^a$ cytotoxic lymphocytes (data not shown). Work with human tumors provides further evidence of the protective effect of tumor associated mucins. In vitro glioma cells produce a glycosaminoglycan covering, which interferes with cell destruction by cytotoxic lymphocytes in a manner which suggests that it inhibits the contact between the target and the effector cell (12). If tumor associated mucins provide a protective barrier against certain immune mechanisms such as CTL and complement mediated antibody lysis, then the mucins are logical targets for a non specific inflammatory reaction such as the one initiated by T(DTH).

Numerous animal models for in vivo immunotherapy have demonstrated the relevance of in vitro generated T cell responses to the inhibition of tumor growth (13-21), where in most cases the tumors involved were induced by viral or chemical means. A tumor model in which the role of conventional transplantation antigens would be minimal was developed using the spontaneous murine mammary adenocarcinoma TA3Ha cell line. The TA3Ha cell line synthesizes a 500,000 m.w. glycoprotein, epiglycanin, which

forms a mucin like glycocalix around the tumor cell. This model was used to establish that T cells respond to tumor associated mucins, particularly to the carbohydrate determinants, and their effect on tumor growth. More specifically we reasoned that the characteristic amplification of a non-specific final effector mechanism of the local DTH reaction may reveal DTH as an ideal anti-tumor immune function , particularly against a tumor where cell surface antigens are masked. When tested, cells primed in vitro with epi were able to delay but not totally inhibit the tumor growth, while cells primed with HSA confered no delay in tumor growth as compared to normal controls. The data suggest that specifically primed cells can confer a delay in the TA3Ha tumor growth in a local Winn assay. Eventually the tumor in the experimentally treated group grew to the same size as that of the control group. This may reflect an inactivation of the primed effector cells once reintroduced to the mouse possibly by removal from the circulation, in the adoptive transfer assay, or by active suppression. Preliminary experiments suport the suppression hypothesis, discussed below. Alternatively, since the TA3Ha tumor is extremely lethal (LD50 In < 10, 22) it is possible that the tumor load may be too high for effective immune elimination and that the tumor simply out grows the adoptively transfered immune response.

The ability of non-specific immune effector mechanisms to inhibit tumor growth is well known. For example, agents which can activate macrophages are able to inhibit tumor growth in an antigen non-specific manner (24). This non-specific mechanism of tumor inhibition is in contrast to a specific immune mechanism involving the recognition of

specific tumor associated antigens. To determine if the mechanism of tumor inhibition observed in the TA3Ha model was dependent on specific antigen recognition or activation of a non-specific effector function, antigen dependence was determined for (1) in vitro induction of effector cells and (2) the in vivo elicitation of the response. The possibility that the tumor growth inhibition was due to an entirely non-specific phenomenon induced during in vitro priming was ruled out by priming with foreign antigen such as HSA. HSA primed cells conferred no inhibition of tumor growth in the Winn assay, thus ruling out the explanation that the inhibition of tumor growth observed previously was due to a non-specific mechanism related to the in vitro priming procedure.

The alternative possibility, that the in vitro epi primed cells are capable of inhibiting any growing tumor was tested as follows. Anti-epi primed cells were injected together with mKSA cells, known to be sensitive to DTH inhibition of growth (see discussion below), and tumor growth was monitored. The results indicate no inhibition of mKSA growth by an epi primed cell population. Inhibition of tumor growth thus requires specific antigen induction and specific recognition of the tumor to inhibit the tumor growth, and appears to be mediated via a classical antigen specific mechanism.

In vitro generation of anti tumor effector cells using s-TAG.

Synthetic carbohydrate antigens made possible the demonstration that the response was directed against a mucin associated carbohydrate determinant. Effector cells were generated against TF-HSA. The primed

cells, when tested in a local Winn assay, were able to inhibit the growth of TA3Ha tumor cells. The inhibition of the tumor growth was mediated by a Thy1+ Lyt1+ Lyt2- cell population. The cell surface phenotype of the anti-tumor effector cell population was shown to be identical to the murine DTH effector cell generated under the same in vitro culture conditions. The similarity of the anti-tumor effector cell population and the cell population mediating the local DTH reaction supports the hypothesis that a DTH reaction triggered by tumor associated mucin determinants can mediate an anti-tumor function. The generation of specific anti-tumor effector cell populations in response to a synthetic carbohydrate antigen becomes particularly important in considering and planning human immunotherapy trials in the cancer clinic.

Adoptive transfer of anti tumor effector cells

The key feature of the immune system, which makes it an ideal candidate for use in cancer therapy is its specificity . Implicit to this is the ability to localize at a specific target. If in vitro manipulations of the immune system are to be of advantage clinically in the treatment of cancer it is necessary that the primed effector cells retain the ability to localize to their target structure. This is an issue of particular concern since long term cultured lymphocytes seem to loose their ability to "home" properly (25). An adoptive transfer assay was used to test the ability of primed cells to inhibit tumor growth following systemic transfer. The primed cells were transfered iv while the tumor challenge was given locally at a subcutaneous site. I found that iv transfered

anti-tumor effector cells were able to to inhibit a subcutaneously growing tumor. Such experiments suggest that a cell mediated response against a tumor associated mucin-like molecule is capable of homing to and inhibiting the tumor growth in a tumor specific manner. Alternatively it is possible that the adoptively transfered cells are not effectors in the tumor growth inhibition and that they do not home to the tumor but rather act by inducing a host mechanism as has been suggested in other systems (16). Since the observed inhibition is tumor specific, the alternative possibility requires that the adoptivly transfered cells induce an effector function with a specificity similar to its own.

Fate of iv transfered cells

It is frequently seen in tumor models that transfer of in vitro primed cells is insufficient to completly eradicate the tumor (17). This has also been shown for the TA3Ha model. This may reflect the extreme lethality of the TA3Ha tumor (23), suppression of the transfered effector cells or systemic clearance of effector cells. The apparent identity between the DTH mediating and the anti-tumor cells was used to further investigate the fate of the in vitro primed cells. Primed cells were transfer iv into the unimmunized host, which were then challenged for DTH at varying times after receiving the primed cells. DTH was elicited with an early antigen challenge (at 3hrs.) whereas no DTH was observed when the challenge was given at a later stage (at 24hrs.). The results support the idea that the primed DTH effector cells are short lived once returned to the in vivo

environment. To determine if the mechanism of this inactivation was via an inability of the cells to home to the local site or via an active suppression of the primed cells the experiment described above was extended. The animals recieved a second iv transfer of primed cells followed by an early (3hr) foot pad challenge for DTH. All the animals tested, except the group which previously received a late (24hr) DTH challenge, responded positively to the DTH challenge. These experiments suggest that a transfer of primed cells if not followed directly by a DTH challenge leads to a state of suppression, such that a second transfer of a DTH effector cell population is suppressed immediately. These preliminary experiments do not rule out other possible explanations for the observed phenomenon but the results are consistent with the hypothesis of active suppression. Further experiments would require the identification of the mechanism of the suppression, for example, isolation of a T suppressor cell population.

In vivo use of sTAGs as potentiators of anti-tumor immunity

The possible use of tumor associated mucins and their synthetic carbohydrate epitopes as cancer vaccines was tested by pre-immunizing mice in situ with epi, TF, Glu-BSA and HSA. Pre-immunization with epi or s-TAG led to a protected state where a significant proportion of immunized animals were able to reject a live tumor challenge. Immunization with Glu-BSA or HSA did not lead to protection against a live tumor challenge. Dose response experiments indicate that the protection generally appeared at doses lower than those leading to antibody formation, suggesting the

effector mechanism may involve cell mediated immunity. However, when the immunized animals were tested for DTH and antibody the protective effect did not correlate well with either. It is possible that the direct DTH assay is not sensitive enough to detect the in vivo primed DTH effector cells. A very small or absent DTH resopnse at the time of assay may develop to an effective response by the time the tumor is rejected. Alternatively it is equally possible that an entirely different mechanism may effect the tumor rejection.

The in vivo dose response to epi suggests that a cell mediated response occurs at a low antigen dose while a humoral response dominates at a higher antigen concentration. Consistent results were obtained in preliminary experiments with a tumor model developed around the mKSA tumor cell line. The mKSA cell line is an SV40 transformed kidney fibrosarcoma of Balb origin and it expresses the virally encoded T-antigen. Experiments conducted in a semi-syngeneic host (Balb x C57) demonstrated that a low dose of antigen preferentially induced a cell mediated immune response while higher doses of antigen led to a humoral response. The protection to the tumor challenge in the mKSA system, similar to the TA3Ha system, does not correlate well with the DTH activity in vivo but in Winn transfer assays protection is seen with the Thy1+ Lyt1+ Lyt2- cell population. Considered together the data presented here suggest that a local DTH reaction can inhibit tumor growth but may not be the only effector mechanism capable of effecting the tumor growth. To suggest that all immunotherapy is equivalent to the induction of an anti-tumor DTH response would be to ignore an enormous volume of literature and to over simplify

the field of tumor immunology. However, it appears that a cell population which mediates a DTH reaction is also capable of inhibiting tumor growth in a specific manner.

The complexity of tumor immunology is again well demonstrated in the following experiments. Excision priming was used to immunize animals against a tumor. Mice were inoculated subcutaneously with live tumor cells, which were allowed to grow to the size of a small tumor nodule and then excised. After allowing some time for recovery the animals were given a live tumor challenge. Preliminary experiments suggest that mice become specifically immune to the antigens associated with the immunizing tumor. Further experimentation by P.A.Bretscher (personal communication) has confirmed these findings. Attempts to determine the class of immune response involved in tumor rejection resulted in inconsistent findings, possibly indicating the absense of a direct correlation between either a DTH or a humoral response and the tumor protective mechanism. Alternatively, the difficulty may be with the assay for the immune function. Any tumor cells present in the animal at the time of the assays may interfere with the detection of any immune functions that is they may bind any tumor specific antibodies and remove the T (DTH) cells from circulation. A similar finding is the lack of detectable immune response to tumor associated antigen in animals with progressive tumor growth. These experiments demonstrate that tumor immunology involves the complex interaction of at least two dynamic systems, the immune system and the growing or regressing tumor.

In conclusion, my studies provide support for the hypothesis that a T (DTH) cell response directed towards a carbohydrate determinant on a tumor

associated mucin-like molecule can inhibit tumor growth. Furthermore, they demonstrate that synthetic carbohydrate analogs of tumor associated antigens can be used to generate an effective class of anti tumor immune reactivity.

This project also reflects the complexity of the immune response to tumor associated antigens. While demonstrating that T cell responses to tumor mucin associated carbohydrate antigens can inihibit tumor growth, the results suggest an in vivo suppression of the adoptively transfered effector cells. The exact mechanism of this appearent suppression remains to be explored. Furthermore the ability of adoptively transfered effector cells to delay tumor growth may have a significant effect on the metastatic spread of a tumor. This also remains to be investigated.

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